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Francis James

Wingless degradation in *Drosophila* imaginal discs

Francis James Marshall

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Division of Mammalian Development,
National Institute for Medical Research,
The Ridgeway, Mill Hill,
London, NW7 1AA

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Signed

Francis James Marshall

Abstract

Wingless is a secreted signalling molecule with multiple roles in patterning developing *Drosophila*. Previous work has shown that in the *Drosophila* embryonic epidermis, regulated degradation controls the distribution of Wingless protein, leading to an asymmetric range. The actual mechanism of Wingless degradation is not currently understood. I have used gain and loss of function experiments to examine the role of the signalling receptors, Dfrizzled-2 and Arrow, in the control of Wingless degradation.

Receptor mediated degradation can be subdivided into three steps: capture, endocytosis and targeting to lysosomes. Dfrizzled-2 is understood to play a key role in capture and indeed when Dfrizzled-2 is overexpressed, Wingless is stabilised at the cell surface. I have shown that Wingless and Dfrizzled-2 also colocalise in endocytic structures and by using mutants of Dfrizzled-2, I have shown that Dfrizzled-2 is actively involved in the endocytosis of Wingless.

Dfrizzled-2 therefore appears to function in the first two steps towards degradation, capture and endocytosis, however it is clearly not sufficient for degradation as when overexpressed, Dfrizzled-2 stabilises Wingless. This suggests that a limiting factor is absent that prevents Wingless captured by DFrizzled-2 from being degraded.

I investigated the possibility that this limiting factor could be Arrow. I have shown that indeed, Arrow brings about the degradation of the Dfrizzled-2-Wingless complex. This activity is specific to Dfrizzled-2, since Arrow does not cause degradation of Wingless stabilised by Dally-like, another Wingless receptor. My results have led to a model where there is a division of labour between the two signalling receptors; Dfrizzled-2 has functions in capture and endocytosis and Arrow, while also contributing somewhat to endocytosis, brings the signal that directs Wingless to lysosomes. Further investigations have been carried out to identify the specific motifs in Arrow that target Wingless to degradation.

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ABBREVIATIONS

A-P	anterior-posterior
AP-2	adaptor protein 2
APC	adenomatous polyposis coli protein
CCP	clathrin coated pit
CCV	clathrin coated vesicle
CDE	clathrin dependent endocytosis
CNS	central nervous system
CKI	casein kinase I
BMP	bone morphogenetic protein
D-V	dorsal-ventral
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
flp	flp recombinase
FRT	flp recombinase target sequence
GFP	green fluorescent protein
GPCR	G-protein-coupled receptor
GPI	glycosyl phosphatidyl inositol
GSK-3β	glycogen synthase kinase-3β
HA	haemaglutinin
HRP	horseradish peroxidase
hs	heat-shock
HSPG	heparan sulfate proteoglycan
LDL	low-density lipoprotein
LRP	low-density lipoprotein-receptor-related protein
MVB	multi-vesicular body
MW	molecular weight
PKA	Protein kinase A
PBS	phosphate buffered saline
PCP	planar cell polarity
PCR	polymerase chain reaction
PS	parasegment boundary
PtdIns[4,5]P_2	phosphatidylinositol 4,5-bisphosphate

RFP	red fluorescent protein
RNAi	RNA interference
RTK	receptor tyrosine kinase
SB	segment boundary
TGFβ	transforming growth factor β
TM	transmembrane
UAS	upstream activating sequence
Vps	vacuolar protein sorting
WIF	wnt inhibitory factor
WT	wild-type

ap	*apterous*
arr	*arrow*
ac	*achaete*
chc	*clathrin heavy chain*
ci	*cubitus interruptus*
Dll	*Distal-less*
dpp	*Decapentaplegic*
dsh	*dishevelled*
en	*engrailed*
fz	*frizzled*
Dfz2	*Drosophila frizzled 2*
Dfz3	*Drosophila frizzled 3*
Dfz4	*Drosophila frizzled 4*
hh	*hedgehog*
ptc	*patched*
sfl	*sulfateless*
sgl	*sugarless*
smo	*smothened*
vg	*vestigal*
wg	*wingless*

CHAPTER 1 – INTRODUCTION

1 CHAPTER 1 - INTRODUCTION

1.1 Secreted signalling molecules pattern developing organisms

The process of animal development begins with a single undifferentiated cell that gives rise to a highly complex adult organism. The body plan of the organism is programmed in detail and as each cell grows it must respond to a number of different inputs from its environment in order for development to proceed correctly. Secreted signalling molecules act to instruct cell fate decisions by the initiation of signalling cascades, which activate the expression of specific genes in the receiving cells. Some secreted signalling molecules act as morphogens, forming concentration gradients and instructing cells to adopt different fates according to the level of signal received. The activity of these signalling molecules must be tightly regulated, as both too much and too little signalling would lead to inappropriate cellular responses and consequently, developmental defects.

The Wnt family of genes are secreted signalling molecules that have multiple roles in the patterning of developing organisms and may act as morphogens. This thesis describes how Wingless, a *Drosophila* Wnt, is degraded through the activity of its receptors.

1.2 Wingless and the Wnt family of genes

The first Wnt gene discovered was the mouse Wnt-1 gene. It was identified as a cellular oncogene, which was activated upon nearby insertion of a mouse mammary tumour virus (Nusse and Varmus, 1982). Wnt genes have since been found throughout the animal kingdom and misregulation of Wnt signalling has been further implicated in cancer (Logan and Nusse, 2004; Nusse, 2005; Taipale and Beachy, 2001). The *Drosophila* segment polarity gene *wingless* is the ortholog of vertebrate Wnt-1 (Cabrera et al., 1987; Rijsewijk et al., 1987).

The first *wingless* mutation was described as a viable allele that results in loss of the wing and duplication of the notum (Sharma, 1973; Sharma and Chopra, 1976). In a genetic screen for zygotic mutations that affect the larval cuticle pattern, null alleles of

wingless were isolated and *wingless* was classified as a segment polarity gene, a class of genes required for the segmentation of the developing embryo and specification of the larval cuticle pattern (Nusslein-Volhard and Wieschaus, 1980). The ventral cuticle of wild-type larvae is characterised by alternating rows of denticle belts and areas of naked cuticle, but in *wingless* mutants the naked cuticle is lost and the cuticle is covered with denticles.

In total, seven Wnt genes have been identified in *Drosophila* and all, apart from Dwnt8, have orthologues in vertebrates (Rubin et al., 2000). Wnts have multiple roles in *Drosophila* development, but, other than that of Wingless, these roles are relatively poorly understood. Dwnt2 is involved in a number of patterning events including the development of the male reproductive tract (Kozopas et al., 1998), direct flight muscle (Kozopas and Nusse, 2002) and the tracheal system (Llimargas and Lawrence, 2001). Dwnt4 is required for cell motility during ovarian morphogenesis (Cohen et al., 2002). DWnt5 is involved in axon guidance in the embryonic nervous system where it acts through the receptor Derailed (Yoshikawa et al., 2003). The expression patterns of Dwnt6 and Dwnt10 have been characterised, although as yet, no function has been attributed to these genes (Janson et al., 2001). Dwnt8, a *Drosophila* Wnt with no vertebrate homolog is expressed weakly in the embryonic nervous system (Llimargas and Lawrence, 2001) but also is of unknown function.

1.3 Wingless/Wnt signalling pathways

Wnt signalling acts through at least three different pathways; the planar cell polarity pathway, the Wnt/Ca^{2+} pathway and the Armadillo/β-Catenin or 'canonical' pathway. In each of these pathways the transmembrane receptor Frizzled acts as a receptor.

1.3.1 The canonical signalling pathway

The canonical Wnt signalling pathway has been well characterised and is highly conserved between organisms (Figure 1.1). The simplest model of canonical Wnt signalling is as follows. In the absence of Wnt, a 'destruction complex' of Axin, GSK3 (known as Shaggy in *Drosophila*) and APC forms, which acts to phosphorylate

cytoplasmic Armadillo/β-Catenin. This results in the proteasome-mediated degradation of Armadillo/β-Catenin.

Wnt signalling is initiated upon Wnt binding to the receptors Frizzled and LRP (Arrow in *Drosophila*). This leads to association of Dishevelled (Dsh) and Axin with the receptor complex, which inhibits formation of the destruction complex. Consequently, this leads to accumulation of cytoplasmic Armadillo/β-Catenin, which then translocates to the nucleus where it associates with TCF to activate the downstream signalling targets (Huelsken and Behrens, 2002; Nusse, 2005) (Figure 1.1).

1.3.2 The planar cell polarity pathway

Epithelial cells exhibit an apical-basolateral polarity, which allows them to carry out functions such as localised secretion of specialised components. In addition to this, cells are sometimes further polarised in the plane of the epithelium, this phenomenon is known as Planar Cell Polarity (PCP). There are many examples of PCP in the animal world including the ordered pattern of feathers and scales on fish and birds and the orientation of cells in the *Drosophila* wing (reviewed in (Mlodzik, 2002). The polarity of the *Drosophila* wing is coordinated by the PCP signalling pathway which functions through Frizzled. Dsh transduces the signal from Frizzled, resulting in the activation of the downstream effectors (Strutt, 2003) (Figure 1.2). In vertebrates Wnts have been implicated in the activation of the PCP pathway (Heisenberg et al., 2000; Tada et al., 2002). However, there is currently no evidence that a Wnt can activate PCP signalling in *Drosophila.*

1.3.3 The Wnt/Ca^{2+} pathway

The Wnt/Ca^{2+} pathway was identified in vertebrates where application of Wnt stimulates intracellular release of calcium (Slusarski et al., 1997). As with the canonical and PCP pathways, Frizzled proteins act as receptors and activate the pathway in a G-protein dependent manner (Wang and Malbon, 2003). However, this pathway has not been shown to be active in *Drosophila.*

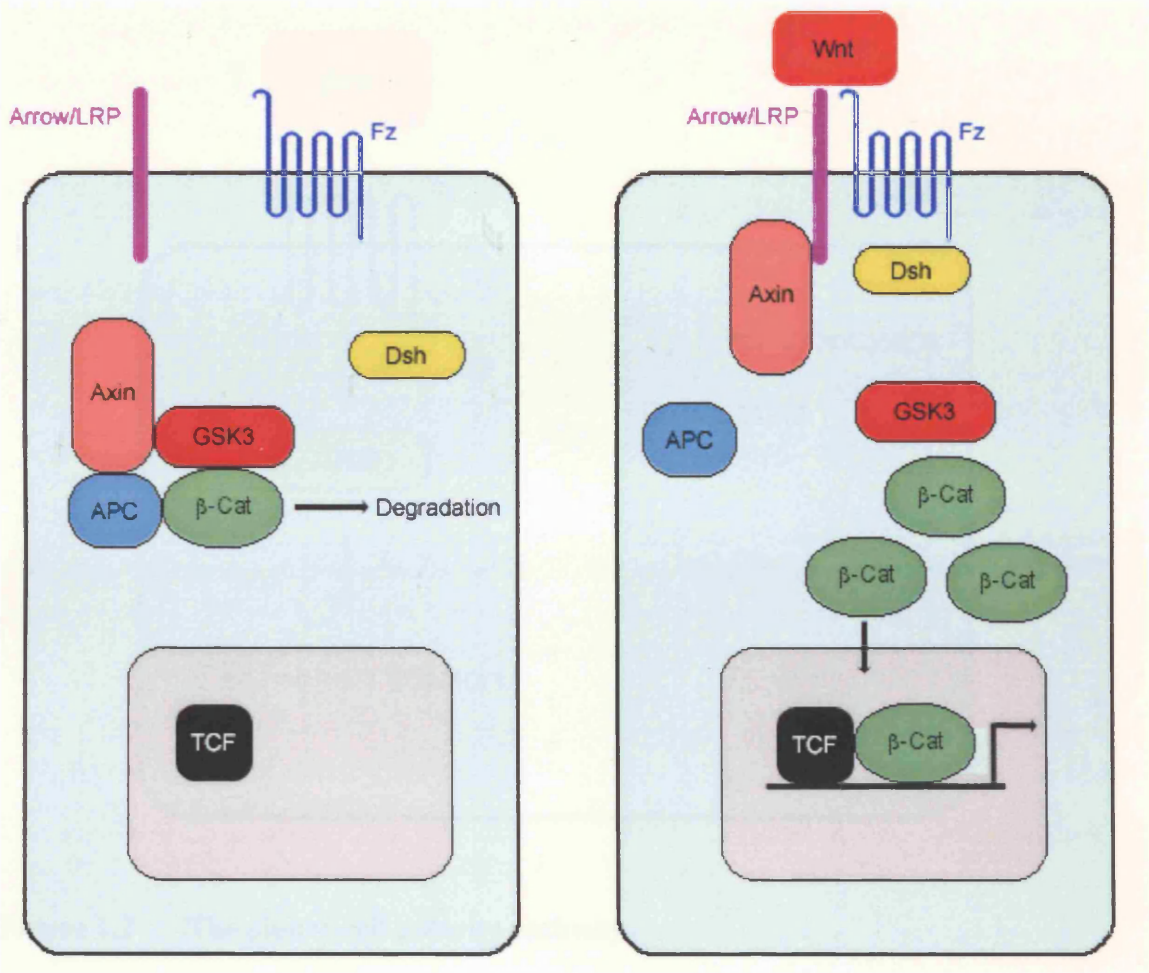


Figure 1.1 The canonical wingless signalling pathway

In the absence of Wnt (left), β-Catenin forms a complex with Axin, APC and GSK3. β-Catenin is phosphorylated by GSK3, resulting in its degradation. Wnt binds to the receptors Frizzled and LRP (right), which recruit Dsh and Axin to the plasma membrane. This leads to the inhibition of β-catenin degradation and its accumulation in the cytoplasm. Stabilised β-catenin then translocates to the nucleus where it interacts with TCF to activate the target genes. (Logan and Nusse, 2004).

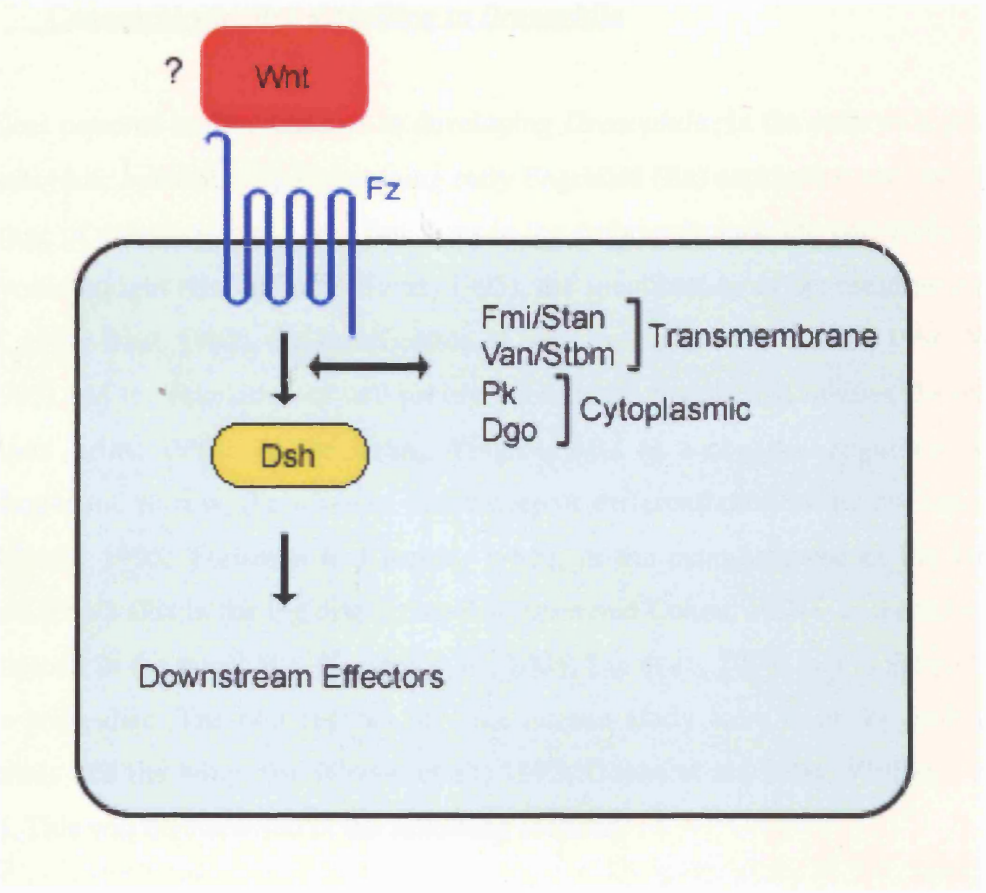


Figure 1.2 The planar cell polarity pathway

The PCP pathway is transduced by Frizzled and requires Dsh. The pathway involves the core planar polarity proteins Flamingo/Starry Night (Fmi/Stan), Van Gogh/Strabismus (Vang/Stbm), Prickle (Pk) and Diego (Dgo), which are thought to form a multiprotein complex. No Wnt ligand has been shown to activate the PCP pathway in *Drosophila*. However, Wnt proteins can activate an analogous pathway in vertebrates (Strutt, 2003).

1.4 Canonical wingless signalling in *Drosophila*

Wingless patterns multiple tissues in developing *Drosophila*. In the embryo it patterns the embryonic epidermis by maintaining early Engrailed (En) expression and specifying the range of naked cuticle. Other functions in the embryo include the patterning of the embryonic midgut (Hoppler and Bienz, 1995), the specification of neuronal precursors (Bhat, 1996; Bhat, 1998), the specification of heart precursors (Park et al., 1996; Wu et al., 1995) and the regulation of cell proliferation in the malphigian tubules (Skaer and Martinez Arias, 1992). In the larva, Wingless acts as a negative regulator of the morphogenetic furrow, the wave of photoreceptor differentiation in the eye disc (Ma and Moses, 1995; Treisman and Rubin, 1995), in the establishment of the Dorso-Ventral (D-V) axis in the leg disc (Diaz-Benjumea and Cohen, 1994), in the activation of apoptosis in the pupal eye (Cordero et al., 2004; Lin et al., 2004) and in the pattering of the wing disc. The two regions of most intense study have been the embryonic epidermis and the wing disc (Couso et al., 1993; Couso et al., 1994; Williams et al., 1993). This will be discussed in the following sections.

1.4.1 Canonical Wingless signalling and the patterning of the embryonic epidermis

The early development of the *Drosophila* embryo involves the subdivision into regions along the Anterior-Posterior (A-P) axis. This process is controlled by the transcription factors encoded by the maternal, gap and pair-rule genes and organises the A-P axis into 14 units named parasegments (Ingham and Martinez Arias, 1992; Sanson, 2001). Within each parasegment, Wingless expression is activated in the most posterior row of cells. Expression of Hedgehog (Hh) and the transcription factor En is activated on the other side of the parasegment boundary (Figure 1.3). Wingless is secreted and prior to stage 10 is distributed symmetrically, 3-4 cells to the anterior and posterior of the producing cells (Figure 1.3). To the posterior, it is required for the maintenance of En and Hh expression. In the absence of Wingless, Hh (Bejsovec and Wieschaus, 1993) and En expression (Bejsovec and Wieschaus, 1993; Cumberledge and Krasnow, 1993; Martinez Arias et al., 1988) decay prematurely.

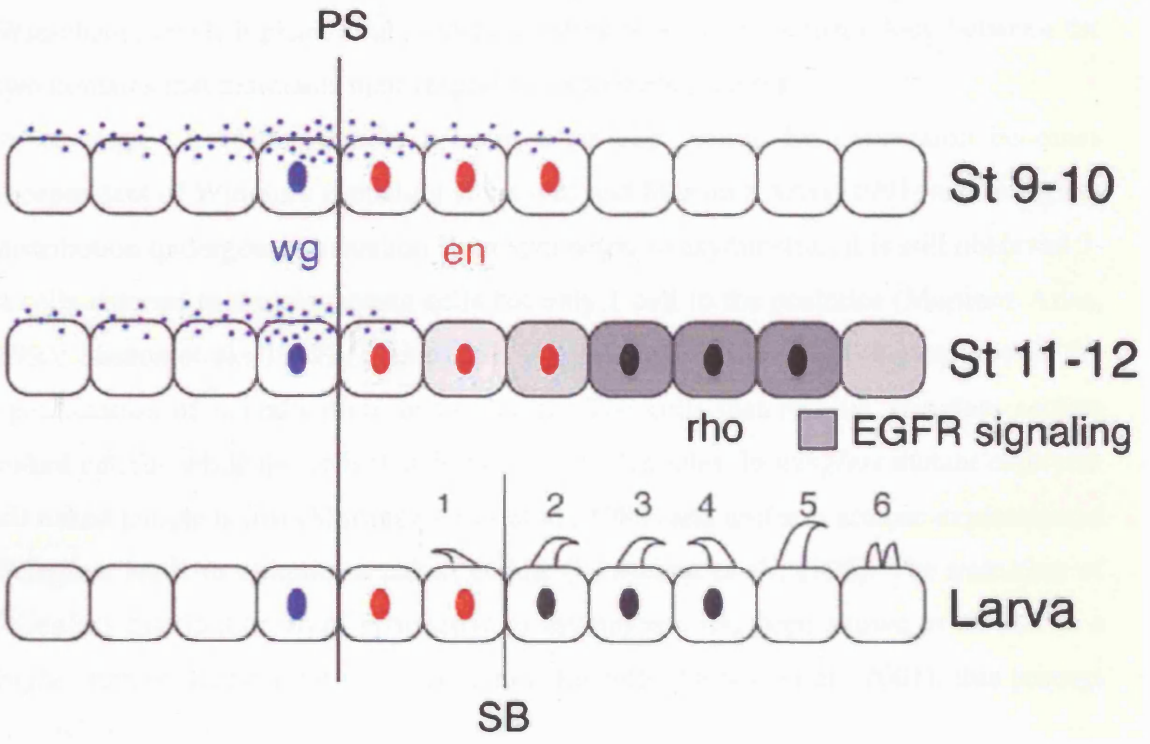


Figure 1.3 Canonical Wingless signalling and the patterning of the embryonic epidermis

Wingless (Wg) and Engrailed (En) are expressed on opposite sides of the parasegment boundary (PS). Prior to stage 10, Wingless spreads symmetrically and acts to maintain Hh and En expression in the posterior cells. Hh, secreted from the engrailed cells, is required for maintenance of Wingless expression.

During stage 10, Wingless distribution becomes asymmetric and is observed 3-4 cells to the anterior but only 1 cell to the posterior of the source. In the larval cuticle, cells that receive Wingless signalling secrete naked cuticle, whereas cells that do not secrete denticles (adapted from Dubois et al., (2001).

Furthermore, Hh is also required for maintenance of Wingless expression (Bejsovec and Wieschaus, 1993; Ingham et al., 1991), creating a positive feedback loop between the two domains that maintains their respective expression patterns.

After stage 11, approximately 6 hours after egg laying, En expression becomes independent of Wingless signalling (Bejsovec and Martinez Arias, 1991) and Wingless distribution undergoes a transition from symmetric to asymmetric, it is still observed 3-4 cells anterior to the expressing cells but only 1 cell to the posterior (Martinez Arias, 1993; Sanson et al., 1999) (Figure 1.3). The primary function of Wingless now is the specification of naked cuticle in the larvae. The cells that receive Wingless secrete naked cuticle, while the cells that do not secrete denticles. In *wingless* mutant embryos, all naked cuticle is lost (Martinez Arias et al., 1988) and uniform ectopic expression of Wingless leads to ubiquitous naked cuticle (Lawrence et al., 1996). The transition of Wingless distribution from symmetric to asymmetric has been shown to be due to a higher rate of degradation in the posterior, En cells (Dubois et al., 2001); this process will be discussed in detail in later sections.

1.4.2 Wingless signalling and the development of the wing imaginal disc

The wing imaginal disc is the structure that gives rise to the adult wing and comprises a single epithelial sheet that becomes subdivided along the A-P and D-V axis (Figure 1.4 a and b). The tissue that gives rise to the wing disc is first recognisable in the embryo as a discrete sac that invaginates from the ectoderm during dorsal closure, 9-10 hours after egg laying, (Bate and Arias, 1991). Some of the cells become stretched and form the peripodial membrane, while the rest of the disc comprises a pseudostratified columnar epithelium with the apical surface facing the lumen (Figure 1.4c).

Direct observation of the wing disc primordia suggests that it arises from 24 cells in the embryo (Bate and Arias, 1991). During larval life, the wing disc proliferates extensively to give rise to a structure comprised of approximately 50,000 cells at the end of the third larval instar (Whittle, 1990).

The parasegment boundary in the embryo gives rise to the boundary that separates the anterior and posterior compartments in imaginal discs. The maintenance of this boundary requires En (Morata and Lawrence, 1975) (Cohen, 1993). The D-V axis is established during the second instar and is marked by the expression of Apterous in the

dorsal compartment (Blair, 1993; Diaz-Benjumea and Cohen, 1993). Wingless is expressed in a dynamic pattern during the second larval instar and acts to specify the wing primordium and the range of apterous expression (Couso et al., 1993; Ng et al., 1996; Williams et al., 1993). The essential role of Wingless is highlighted by the observation that flies that lack Wingless during this stage fail to produce wings (Williams et al., 1993), the phenotype that originally gave *wingless* its name (Sharma and Chopra, 1976). Furthermore, ectopic expression of Wingless can lead to duplication of the wing (Ng et al., 1996).

During the third larval instar, the Notch signalling pathway, activated by the ligand Serrate, induces Wingless expression at the D-V boundary of the disc (Diaz-Benjumea and Cohen, 1995) (Figure 1.4d). Wingless signalling activity is required for the formation of the wing margin, (Couso et al., 1994). Loss of Wingless signalling in this region results in a failure to specify margin cell fates (Couso et al., 1994) and notching of the wing blade (Baker, 1988). In the cells adjacent to the D-V boundary, Wingless signalling induces the formation of the specialised bristles found at the margin of the adult wing (Blair, 1993; Couso et al., 1994; Phillips and Whittle, 1993) (Figure 1.4a).

A further function of Wingless in the disc is in the regulation of disc size. *wingless* mutant discs show reduced mass, a finding that suggests that Wingless promotes growth in the wing disc (Serrano and O'Farrell, 1997). Cells lacking Wingless signalling often die (Williams et al., 1993) and recent work has suggested that Wingless does not regulate disc growth by promoting proliferation, but instead but promoting survival (Johnston and Sanders, 2003).

Wingless is secreted on the apical face of the epithelium and forms a steep, symmetrical gradient on the basolateral surface. It is detected up to 10-15 cells away from the source (Strigini and Cohen, 2000). It has been suggested that Wingless acts as a morphogen, directly regulating cell identity in a concentration dependent manner (Neumann and Cohen, 1997; Zecca et al., 1996).

Evidence supporting this idea comes from studies that examined the effect of Wingless signalling on the expression of putative Wingless target genes. Three nested domains of expression are observed in the wild-type disc. Achaete and Neuralized are expressed in narrow domains, adjacent to the source of Wingless, Distal-less (Dll) in a wider domain and Vestigal in the widest domain. These domains of expression have been suggested to represent distinct threshold responses to Wingless signalling (Neumann and Cohen,

1997)(Figure 1.4d). Indeed, ectopic expression of Wingless leads to the induction of expression of these genes in concentric domains surrounding the clones, in a similar manner to that observed surrounding the endogenous source of Wingless (Neumann and Cohen, 1997; Zecca et al., 1996). Furthermore, reducing the dosage of Wingless at specific times during development, using a temperature sensitive allele of *wingless*, results in reduced domains of each of these target genes (Neumann and Cohen, 1997). Expression of a membrane tethered form of Wingless fails to activate the targets at a distance, suggesting that Wingless signalling is not transmitted by a signal relay mechanism but acts directly to specify cell fates (Zecca et al., 1996).

Together, these findings have lead to the conclusion that Wingless acts as a morphogen in the wing disc. However, it has been argued that Wingless does not fulfil the criteria in needed to be classified as a classical morphogen (Martinez Arias, 2003). The author argues that the experiments described above do not take into account parameters such as the growth of the wing and the order of onset of gene expression. As explained previously, the expression pattern of Wingless is dynamic in the developing disc, culminating in the formation of the D-V boundary stripe during the third instar. However, both expression of Achaete and Dll is activated prior to the formation of the D-V stripe, suggesting that Wingless is not required for the initiation of their expression. Therefore, Wingless, rather than playing an instructive role, could mainly be required for the maintenance of expression patterns and consequently cannot be classified as a classical morphogen (Martinez Arias, 2003).

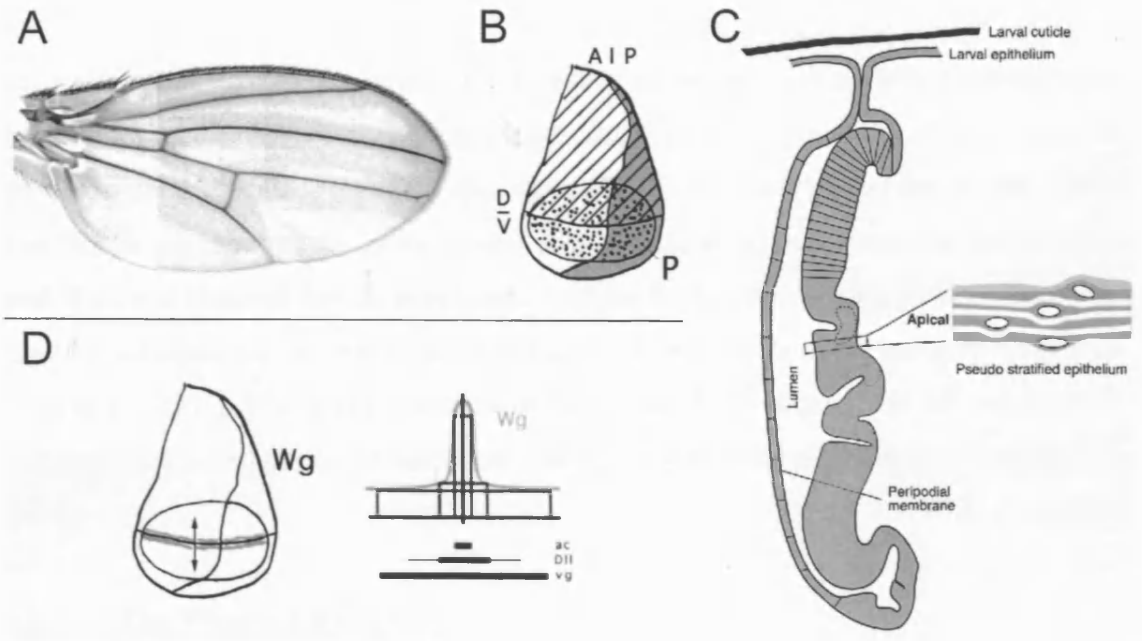


Figure 1.4 Patterning of the *Drosophila* wing

(A) The adult wing of *Drosophila* comprises a single epithelial sheet folded over upon itself to make the dorsal and ventral surfaces. Specialised bristles line the wing margin.
(B) Schematic of the larval wing disc showing the posterior compartment in grey, the dorsal compartment (striped) and the A-P and D-V boundaries.
(C) Cross section of the wing disc. The wing disc is a folded, sac-like structure, continuous with the larval epithelium. It comprises the peripodial epithelium and the pseudostratified columnar epithelium, which faces the disc lumen.
(D) Wingless is produced at the D-V boundary and forms a steep gradient that activates the targets *achaete* (ac) *Distal-less* (Dll) and *vestigal* (vg).
(Lawrence, 1992; Strigini and Cohen, 1999).

1.5 Wnt Signalling in other species

In vertebrates, 20 Wnt genes have been identified (Wnt genes homepage, http://www.stanford.edu/~rnusse/wntwindow.html) and they regulate a diverse range of developmental processes (Wodarz and Nusse, 1998). For example, in the mouse, Wnt1 and Wnt3a are required for development of the CNS, Wnt3 is required for gastrulation and Wnt5a is required for the development of the limbs (Wodarz and Nusse, 1998). In the frog *Xenopus laevis*, Wnt11 is required for the formation of the primary body axis (Tao et al., 2005). Wnt genes also exist in the nematode *C. elegans* and are required for the asymmetric cell divisions that occur during early development (Wodarz and Nusse, 1998).

1.6 The Wingless Receptors

1.6.1 Frizzled

The identity of the primary signalling receptors for Wingless remained elusive for a number of years. However, in 1996 Bhanot and colleagues identified the seven-pass transmembrane protein Dfrizzled2 (Dfz2) as a Wingless receptor in *Drosophila*. Upon application of Wingless to *Drosophila* S2 cells transfected with Dfz2, intracellular Armadillo levels are elevated, signifying the activation of Wingless signalling. Furthermore, Dfz2 transfected S2 cells exhibit Wingless binding, which is absent in untransfected cells. Following this finding, further analysis confirmed that Frizzled proteins act as Wnt receptors (He et al., 1997; Kennerdell and Carthew, 1998; Yang-Snyder et al., 1996)

1.6.1.1 Structure of Frizzled proteins

Frizzled proteins are characterised by the presence of a signal peptide at the N-terminus, a cysteine rich domain (CRD), a divergent region of 40-100 hydrophilic residues, seven transmembrane domains and a cytoplasmic tail (Wang et al., 1996; Wodarz and Nusse, 1998) (Figure 1.5). They vary in length from 525 amino acid residues (Cfz1) to 709 amino acid residues (mFz6) (Wang et al., 1996).

The signal sequence is present in all Frizzled proteins but is not conserved at the level of primary sequence (Wang et al., 1996). The CRD domain is comprised of 120-125 residues containing 10 conserved cysteines (Bhanot et al., 1996), which form disulphide bonds (Dann et al., 2001). The crystal structure of mFz8 CRD is mainly α-helical in nature and contains a novel protein fold (Dann et al., 2001). The CRD has been identified as a ligand-binding site in Frizzled. A truncated form of Dfz2, that lacks the CRD domain, fails to bind Wingless (Bhanot et al., 1996).

In crystals, the CRD of mFz8 forms dimers. However, in solution it exists as monomers, so the biological significance of dimerisation remains questionable. Interestingly however, when 2 differently tagged Fz8CRDs are coexpressed in cells, an increase in association is observed upon application of Xwnt8 (Dann et al., 2001), suggesting that Frizzled CRD dimerisation could occur on ligand binding. However, the Fz-Wnt complex has yet to be molecularly defined. Comprehensive mutational analysis in this same study identified the residues in the Frizzled CRD that are required for Wnt binding. These were mapped to a single surface on the crystal structure (Dann et al., 2001).

The hydrophilic region following the CRD is highly divergent between family members and varies from 40 to 100 amino acid residues in length. This region is likely to form an extended linker between the CRD and the transmembrane domain (Wang et al., 1996).

Following the hydrophilic region are seven highly conserved, hydrophobic regions of 20-25 residues in length separated by short hydrophilic domains. These domains are predicted to form transmembrane domains similar to those present in G-protein coupled receptors (GPCRs). The sequence homology to GPCRs is low. Nevertheless, a recent study found that Frizzled activation of both the canonical Wnt and the PCP pathway occurs in a G-protein dependent manner (Katanaev et al., 2005). The cytoplasmic domain of Frizzled proteins are highly divergent and vary in length from 25 to 200

amino acid residues (Wang et al., 1996). A conserved KTxxxY motif located 2 residues after the 7th transmembrane domain is required for the activation of Wnt/Frizzled signalling (Cong et al., 2004; Umbhauer et al., 2000) and it was shown to bind PDZ domains (Wong et al., 2003).

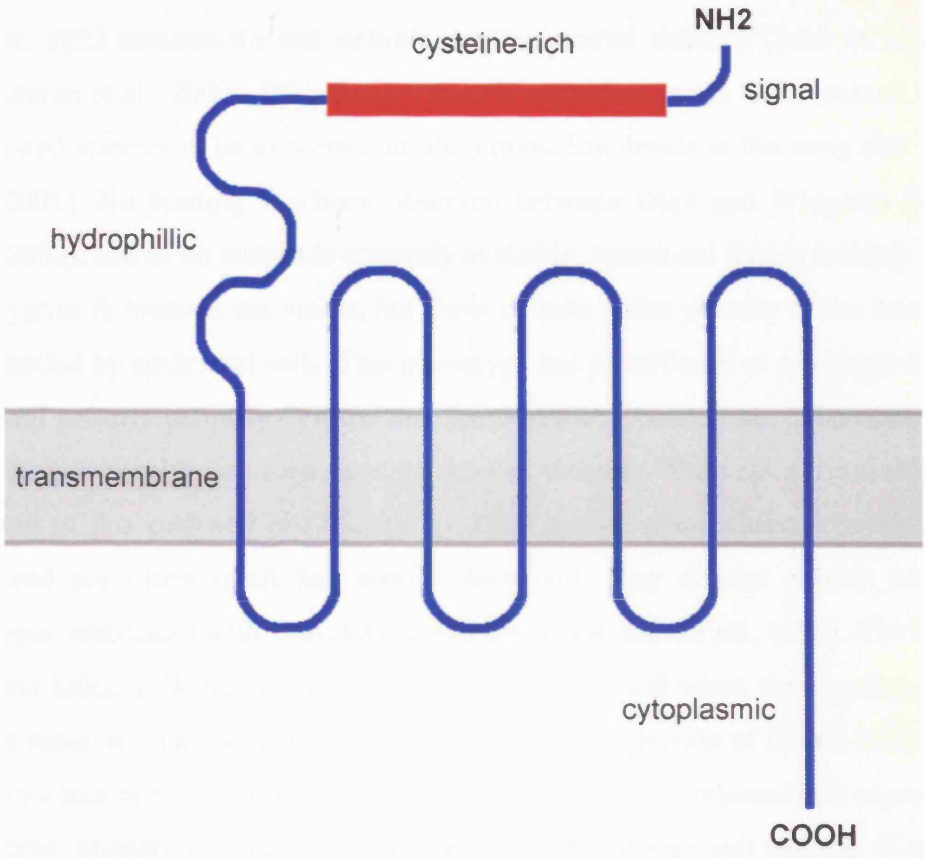


Figure 1.5 Structure of Frizzled proteins

Frizzled family members are transmembrane proteins characterised by an N terminal signal peptide, an extracellular cysteine-rich domain, a hydrophilic region followed by seven transmembrane domains and a cytoplasmic tail (Wodarz and Nusse, 1998).

1.6.1.2 Function of Frizzled proteins in Wingless signalling

Four Frizzleds have been identified in *Drosophila* (Rubin et al., 2000). Frizzled (Fz) and Dfz2 act as Wingless receptors (Bhanot et al., 1999; Bhat, 1998; Chen and Struhl, 1999; Kennerdell and Carthew, 1998). Dfrizzled3 (Dfz3) possibly functions as an attenuator of Wingless signalling, a suggestion based on its ability to modify some *wingless* mutants (Sato et al., 1999). Wingless binds Dfz3 (Wu and Nusse, 2002), however, *Dfz3* mutants do not exhibit developmental defects (Sato et al., 1999; Sivasankaran et al., 2000). Dfrizzled4 (Dfz4) is expressed in the CNS (central nervous system) and appears to be expressed at ubiquitous, low levels in the wing disc (Janson et al., 2001). No binding has been observed between Dfz4 and Wingless (Wu and Nusse, 2002), and as no mutant is currently available, functional data is lacking.

Homozygous *fz* mutants are viable, but show defects in the polarity of the bristles and hairs secreted by epidermal cells. This phenotype has identified *fz* as a component of the planar cell polarity pathway (Vinson and Adler, 1987; Zheng et al., 1995) (see section 1.3.2). As previously described, it is not known whether Wingless plays a role in the activation of this pathway in *Drosophila*. *Dfz2* mutant flies exhibit a developmental delay, and are often small and sterile. However, they do not exhibit any overt phenotypes associated with Wingless signalling (Chen and Struhl, 1999). The function of Fz and Dfz2 in Wingless signalling is only uncovered when the function of both genes is removed. This was first shown by RNAi. Co-injection of fz and Dfz2 dsRNA results in a loss of naked cuticle in the larvae and a failure to maintain En expression in the embryo, phenotypes similar to those observed in *wingless* null mutants (Kennerdell and Carthew, 1998). The generation of *fz, Dfz2* double mutants further demonstrated the redundancy of Fz and Dfz2. Embryos lacking both maternal and zygotic *fz* and *Dfz2* exhibit cuticle phenotypes identical to that of *wingless* mutants, fail to maintain En and exhibit defects in heart and gut morphogenesis (Bhanot et al., 1999; Chen and Struhl, 1999). Furthermore, wing disc cells that lack fz and Dfz2 fail to activate Wingless targets (Chen and Struhl, 1999). Epistasis experiments show that even in the presence of ectopic Wingless, *fz Dfz2* mutants do not activate Wingless signalling, whereas expression of an activated form of the downstream effector Armadillo in the mutants can trigger Wingless signalling (Bhanot et al., 1999). Together, these experiments

confirm that Frizzled proteins are essential components of the Wingless signalling pathway and Fz and Dfz2 can act redundantly to transduce the Wingless signal.
Due to its high affinity for Wingless (Rulifson et al., 2000; Wu and Nusse, 2002), Dfz2 could shape the Wingless gradient and two pieces of evidence have been used to suggest a mechanism by which this could occur. Firstly, when ectopically expressed, Dfz2 stabilises Wingless (Cadigan et al., 1998). Secondly, Dfz2 expression is repressed by Wingless signalling in the wing disc, resulting in inverse patterns of expression of Wingless and Dfz2 (Cadigan et al., 1998). In a model proposed by Cadigan et al., (1998), Wingless close to the source would have a high turnover, as Dfz2 is only present at low levels. Further from the source, higher levels of Dfz2 would stabilise the small amounts of Wingless that reach this area and protect it from degradation. This model would lead to a stable, steep gradient of Wingless in the disc, as is exhibited by Wingless. It has been suggested that Dfz2 could protect Wingless by titrating a putative extracellular protease (Eldar et al., 2003), although currently no such molecule has been identified.

1.6.2 Arrow/LRP

The transmembrane protein Arrow and its vertebrate homologs LRP5 and LRP6 were recently identified as essential components of the canonical Wingless signalling pathway (Wu et al., 1995). Arrow and LRP5/6 are members of the low-density lipoprotein receptor-related (LRP) family that form part of the LDL receptor super-family. LDL receptors function as endocytic receptors and have a wide range of cellular functions including binding and uptake of lipoprotein, proteases, protease inhibitors, signalling molecules, toxins and antibiotics (Herz and Bock, 2002; Nykjaer and Willnow, 2002; Wei et al., 2006).

1.6.2.1 Structure of LDL proteins and Arrow/LRP

LDL proteins are characterised by the presence of a number of domains. The extracellular domain contains a signal peptide, cysteine-rich LDL binding repeats of approximately 40 amino acids (which form the ligand binding domain) and cysteine rich EGF repeats (required for the pH-dependent release of ligands in endosomes

(Rudenko et al., 2002)), separated by spacer domains of ~50 amino acids containing the YWTD motif. Following these motifs are a single transmembrane domain and a cytoplasmic tail, which generally with generally at least one NPXY motif, which is involved in the internalisation of the receptor (Nykjaer and Willnow, 2002).

Arrow exhibits 71% similarity and 40% identity to LRP5 and 6 (Wehrli et al., 2000) and together, these proteins form a distinct group of LRPs. Arrow contains a signal peptide at the N-terminus, four EGF-like repeats preceded by six YWTD spacer domains and three LDL repeats (Figure 1.6). Following this is a putative transmembrane domain and a Proline and Serine rich cytoplasmic tail of 209 amino acid residues in length (Figure 1.6). Arrow and LRP5/6 do not contain the NPXY motif (Wehrli et al., 2000). The cytoplasmic tail also contains 5 conserved PPP(S/T)P motifs, which are required for the activation of Wnt signalling (He et al., 2004; Tamai et al., 2004).

Figure 1.6 Structure of Arrow

Arrow is a transmembrane receptor of the LRP family. It contains a signal peptide at the N-terminus; four EGF-like repeats separated by spacer domains (LY) and three LDL repeats. Arrow contains a single transmembrane domain (TM), followed by a Proline/Serine rich cytoplasmic tail. (Structure generated using SMART (http://smart.embl-heidelberg.de/)).

1.6.2.2 Arrow/LRP Function

The first link of Arrow and LRPs to Wnt signalling was established in three independent studies with *Drosophila*, mouse and *Xenopus* (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). In *Drosophila*, *arrow* was identified based on its embryonic phenotype, embryos lacking both maternal and zygotic *arrow* fail to maintain En expression and exhibit a denticle phenotype identical to that of *wingless* mutants (Wehrli et al., 2000). Furthermore, mutant clones of *arrow* in the wing disc fail to activate the Wingless target gene *Dll*. Ectopic expression of Dsh is able to activate Wingless signalling in the absence of Arrow, indicating that Arrow is upstream of Dsh in the canonical Wingless signalling pathway (Wehrli et al., 2000). This study also found that Arrow does not function in the PCP pathway, as *arrow* mutant cells in the wing exhibit normal polarity (Wehrli et al., 2000). In the mouse, LRP6 mutants exhibit severe developmental defects including loss of limb structures, malformation of the urogenital system, truncations of the body axis, and mid-hindbrain defects, phenotypes similar to those caused by mutations in a number of Wnt pathway members. Furthermore, removal of one copy of LRP6 enhances the vestigal tail phenotype exhibited by a hypomorphic mutation in Wnt3a (Pinson et al., 2000). In *Xenopus*, injection of LRP6 mimics the axis duplication phenotype exhibited when Wnt is injected and leads to the activation of Wnt target genes. A truncated form of LRP6 which lacks most of the cytoplasmic tail is not able to activate signalling, suggesting that essential signalling motifs reside in the tail (Tamai et al., 2000). Further analysis has identified the PPP(S/T)P motifs present in the Arrow/LRP5/6 cytoplasmic tails as essential signalling motifs (Tamai et al., 2004).

Recently, the kinases required for LRP6 phosphorylation have been identified. GSK3 phosphorylates the PPP(S/T)P motif. This promotes phosphorylation of a second conserved site three amino acids downstream of the PPPSP phosphorylation site by Caesin Kinase I γ (CK1γ) leading to the activation of Wnt signalling (Davidson et al., 2005; Zeng et al., 2005).

1.6.3 Proteoglycans and Wnt Signalling

Heparan Sulphate Proteoglycans (HSPGs) are abundant cell surface molecules that form part of the extracellular matrix (Lander and Selleck, 2000). HSPGs consist of a protein core and a number of heparan sulphate glycosaminoglycan chains. There are two major groups of HSPGs, those that are linked to the membrane by a GPI anchor (glypicans) and those that have a transmembrane domain (syndecans) (Bernfield et al., 1999). Glypicans have been implicated in signalling by a number of secreted signalling molecules including Wingless, Hh and Decapentaplegic (Dpp) (Baeg et al., 2001; Desbordes and Sanson, 2003; Fujise et al., 2003; Jackson et al., 1997).

HSPGs were first implicated in Wingless signalling by the observation that Wingless binds heparin and heparan sulphate in S2 cells and that the removal of these molecules results in an impairment in Wingless signalling (Reichsman et al., 1996). This finding was supported by the discovery of two enzymes, Sugarless (Sgl) and Sulfateless (Sfl), which are required for Wingless signalling in *Drosophila*. *sgl* encodes the *Drosophila* homologue of vertebrate UDP-glucose dehydrogenase, which is required for the synthesis of heparan sulphate and *sgl* mutants resemble *wingless* mutants (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997). *sfl* encodes an N-deacetylase/N-sulphotransferase also required for the biosynthesis of HSPGs. *sfl* mutant embryos also have a cuticle phenotype identical to that of *wingless* mutant embryos and exhibit reduced Wingless signalling (Lin and Perrimon, 1999).

These discoveries lead to the implication of two glypicans in Wingless signalling, the first one identified was Dally. Embryos that have Dally function reduced through RNAi injections have been reported to exhibit weak segment polarity phenotypes (Lin and Perrimon, 1999; Tsuda et al., 1999). However, this phenotype could not be replicated in subsequent experiments (Desbordes and Sanson, 2003). The second glypican implicated in Wingless signalling was Dally-like. Dally-like RNAi injection generates a segment polarity phenotype in the embryo (Baeg et al., 2001), however a further study suggested that this phenotype may be a result of the effect of Dally-like on Hh, rather than Wingless signalling (Desbordes and Sanson, 2003). Genetic analysis of the role of glypicans in Wingless signalling was hampered by the lack of null mutants, however these have recently become available. *dally* null mutants are viable and fertile, confirming the RNAi phenotype of Desbordes and Sanson (2003). In the adult wings,

dally mutant flies exhibit notches at the margin (Franch-Marro et al., 2005), suggesting a reduction in Wingless signalling. *dally-like* zygotic mutants can survive to adulthood, however, *dally-like* maternal and zygotic mutants exhibit a segment polarity phenotype (Kirkpatrick et al., 2004) (Franch-Marro et al., 2005). This phenotype is enhanced by the absence of Dally, suggesting that Dally could play a role in transducing the Wingless signal, but in its absence, Dally-like is able to compensate (Franch-Marro et al., 2005). *dally dally-like* double mutants resemble *wingless hh* double mutants, suggesting that the glypicans are required for the function of both of these signalling molecules in *Drosophila* embryos (Franch-Marro et al., 2005).

Due to their effects on multiple signalling pathways, further analysis of Dally and Dally-like function in the embryo has proved difficult; therefore researchers have turned to the wing disc.

In the wing disc, ectopic expression of Dally-like leads to accumulation of Wingless (Baeg et al., 2001). Interestingly, under these conditions, there is a reduction in the activation of Wingless target genes close to the source (the high level targets) but an increase in activation further away from the source (the low level targets) (Franch-Marro et al., 2005; Kirkpatrick et al., 2004). This finding correlates with the observation that discs deficient for Dally-like, either by RNAi or by classical genetic mutants, exhibit increased Wingless signalling close to the source, and reduced signalling further away (Franch-Marro et al., 2005; Kirkpatrick et al., 2004; Kreuger et al., 2004). This finding suggests that at high Wingless levels, Dally-like limits signalling, potentially by promoting transport, and at low Wingless levels Dally-like promotes signalling. A role for Dally-like in transport of Wingless is further suggested by the observation that clones mutant for *dally-like* exhibit reduced Wingless protein levels (Franch-Marro et al., 2005). Interestingly, in Dally-like overexpression clones, where Wingless signalling is reduced inside the clone, ectopic Wingless signalling is occasionally observed outside the clone (Franch-Marro et al., 2005). Suggesting that the role of Dally-like is to sequester Wingless from the signalling receptors in the cell to which it is bound, but to present Wingless to the receptors on the adjacent cells.

The current model for glypican function in Wingless signalling is as follows; when Wingless binds Dally-like, it is prevented from accessing the signalling receptors in the cell to which it is bound, however it can pass Wingless on to the adjacent cell and consequently, promotes Wingless transport. Dally, while not essential for Wingless

signalling, appears to play a positive role in signal transduction, possibly by binding Wingless and presenting it to the signalling receptors (Franch-Marro et al., 2005).

1.6.4 Derailed

Derailed is an atypical receptor tyrosine kinase (RTK), which was recently identified to act as a Wnt receptor during axon guidance in the *Drosophila* nervous system (Yoshikawa et al., 2003). The kinase domain of Derailed has mutations in key residues and lacks catalytic activity (Halford and Stacker, 2001).

Derailed contains a WIF domain that serves as the binding site for Wnt5, apparently the only *Drosophila* Wnt that can bind to Derailed (Yoshikawa et al., 2003). In *Drosophila* the mechanism of Wnt signalling through Derailed remains unclear, although it appears to function through a non-canonical process. Genetic analysis suggests that neither Fz, Dfz2 nor Dsh are required for Derailed-mediated signalling (Yoshikawa et al., 2003).

Interestingly however, the mammalian homolog of Derailed, Hyk, can bind Wnt-1 and Wnt3a and also interacts physically with Frizzled and Dsh. In cell culture assays, transfection of Hyk and Wnt3a leads to activation of a TCF reporter construct in a Dsh-dependent manner, suggesting that Wnt signalling through Hyk can activate the canonical Wnt signalling pathway. Hyk is required in only a few developmental processes, suggesting that it acts only in specific tissues to mediate Wnt signalling (Halford and Stacker, 2001)

In *C. elegans*, the *derailed* homolog LIN-18 is required for vulval development. Genetic analysis suggests that it mediates Wnt signalling in a parallel pathway to Wnt-Frizzled signalling, and LIN-17 (a *C. elegans* Frizzled) is not required for LIN-18 activity. Downstream components have not been identified and it remains to be seen whether Dsh interacts with LIN-18 (Inoue et al., 2004)

These findings suggest that Derailed can transduce Wnt signalling but it does not appear to transduce Wingless signalling in *Drosophila*. However, two further *hyk* homologs, *doughnut* and *derailed-2*, exist in *Drosophila*. Whether they can transduce Wingless signalling is not known.

1.7 How is signalling activated by the receptors?

1.7.1 Ligand binding

The mechanism by which the signalling receptors transduce the Wnt signal has been an area of intense investigation and several pieces of evidence have shed light on this process. Ligand binding is the first step and a large body of evidence suggests that this is primarily mediated by the CRD of Frizzled proteins (Bhanot et al., 1996; Dann et al., 2001; Hsieh et al., 1999b; Rulifson et al., 2000; Sato et al., 1999; Wu and Nusse, 2002). The affinities of the Frizzled CRDs have been measured and the Dfz2 CRD exhibits the highest affinity having an approximately 10 fold higher affinity for Wingless than the CRDs of Fz or Dfz3 (Table 1.1), the Dfz4 CRD does not appear to bind Wingless.

	Wg Binding
Fz	$4.51 \pm 0.25 \times 10^{-8}$M
Dfz2	$5.44 \pm 0.26 \times 10^{-9}$M
Dfz3	$5.25 \pm 0.19 \times 10^{-8}$M
Dfz4	NB

NB – No detectable binding

Table 1.1 Binding affinities between Wingless and Drosophila Frizzleds CRDs

Kds were calculated by incubating S2 cells stably expressing the membrane tethered Neurotactin-Wg fusion protein with conditioned medium containing a fusion of the CRDs of Fz, Dfz2, Dfz3 or Dfz4 to Alkaline Phosphatase (AP). No binding was detected between Wg and Dfz4 (Wu and Nusse, 2002).

Despite the above observations, a recent study suggested that the CRD of Frizzled is dispensable for Wingless signalling. Ectopic expression of forms of Fz and Dfz2 that lack the CRD (FzΔCRD and Dfz2ΔCRD respectively) were observed to extensively rescue the *fz, Dfz2* mutant phenotype (Chen et al., 2004). A subsequent study was unable to fully replicate the rescue using FzΔCRD but Wingless signalling was observed to be partially restored in *fz, Dfz2* mutants in the presence of FzΔCRD,

suggesting that Wingless signalling can be activated in the absence of the CRD (Povelones and Nusse, 2005). Interestingly, a form of Fz where the CRD is replaced with Wingless itself, constitutively activates signalling, a finding that suggests that the function of Wingless binding to the CRD may be to bring Wingless is close proximity to other domains of the receptor, which would trigger signal transduction.

A number of co-immunoprecipitation studies have reported Wnt binding to Arrow/LRP5/6 (Itasaki et al., 2003; Kato et al., 2002; Liu et al., 2003; Mao et al., 2001; Tamai et al., 2000; Tamai et al., 2004). However, a biochemical study failed to detect any interaction between Arrow and Wingless (Wu and Nusse, 2002), this could possibly due to the weakness of the interaction. The discovery that Wnt proteins are lipid modified by the addition of a palmitate (Willert et al., 2003) raised the possibility that Arrow/LRP5/6, due to their relationship with lipoprotein receptors, could bind lipid modified Wnts.

1.7.2 Transduction after Ligand Binding

The canonical Wnt signalling pathway (Figure 1.1) is activated by inhibition of the complex that degrades cytoplasmic Armadillo/β-catenin. Dsh is an essential component for the downstream signalling events and is suggested to act as a link between the receptors and the degradation complex.

The mechanism of signal transduction after ligand binding appears to depend on the close association of LRP and Frizzled. The mFz8 CRD forms a Wnt-dependent complex with the LRP5 and LRP6 extracellular domains (Semenov et al., 2001; Tamai et al., 2000) and the formation of artificial complexes between Arrow/LRP and Frizzled, either by creating fusion proteins, or by using inducible oligomerisation strategies, results in the activation of signalling in a ligand independent manner (Cong et al., 2004; Tolwinski et al., 2003). Together, these findings suggest a model whereby Wnt acts as a bridge that leads to the association of Frizzled and LRP. However, so far, Wnt induced oligomeristaion of endogenous Frizzled and LRP has not yet been observed (Wu and Nusse, 2002).

Motifs in the cytoplasmic tails of Frizzled and Arrow/LRP mediate signal transduction to the cytoplasmic components of Wnt signalling. The KTxxxY motif in the

cytoplasmic tail of Frizzleds is required for the activation of Wnt signalling and acts as a binding site for the PDZ domain of Dsh (Cong et al., 2004; Umbhauer et al., 2000). Dsh can bind Axin Wnt triggers the recruitment of Dsh and Axin to the plasma membrane (Cliffe et al., 2003; Cong et al., 2004). The cytoplasmic tail of Arrow/LRP contains CK1 consensus sites and PPPSP motifs. Following phosphorylation by CK1γ and GSK3, these sites act as docking sites for Axin, which possibly leads to its degradation (Davidson et al., 2005; Mao et al., 2001; Tamai et al., 2004; Tolwinski et al., 2003; Zeng et al., 2005). This prevents the formation of the destruction complex, Armadillo is stabilised and translocates to the nucleus to activate signalling.

In summary, the current model for signal transduction by the receptors is as follows: Wnt binding to Frizzled and LRP stimulates the association of the two receptors. Frizzled recruits Dsh which brings with it Axin to the plasma membrane. The phosphorylation of Arrow/LRP acts as a docking site for Axin, thus stabilising its translocation to the plasma membrane and resulting in the inhibition of the destruction complex.

1.8 Regulation of Wnt Signalling

As stated in section 1.1, excess signalling by secreted signalling molecules must be prevented as it can lead to developmental abnormalities and tumourigenesis in adults (Freeman, 2000). A number of mechanisms are used in order for a cell to limit its response to a secreted signal. These include: the modulation of receptor levels present in the receiving cells, the regulation of ligand transport, the expression of secreted inhibitors and degradation of the ligand by extracellular proteases or intracellularly. I will now discuss the methods utilised to limit Wnt/Wingless signalling.

1.8.1 Secreted Inhibitors of the Canonical Wnt Signalling Pathway

A number of secreted factors have been identified that act as inhibitors of Wnt signalling (Kawano and Kypta, 2003). These molecules either act by binding directly to Wnt proteins, in the case of secreted Frizzled-related proteins (sFRP), Wnt inhibitory factor-1 (WIF-1) and Cerberus, or by binding to components of the receptor complex, in the case of the Dickkopf family and Wise.

sFRPs share homology to the CRD of Frizzled proteins and are able to bind Wnts and inhibit Wnt signalling in *Xenopus* embryos and cell culture (Wang et al., 1997). So far no sFRPs have been identified in *Drosophila*, however, mammalian sFRPs can bind Wingless and inhibit its activity (Uren et al., 2000). The only sFRP identified in invertebrates so far is suSFRP1 from the sea urchin (Illies et al., 2002), however it is not known whether it has the ability to inhibit Wnt signalling. WIF-1 can also bind Wnt and inhibit its function in *Xenopus* embryos and cultured cells (Hsieh et al., 1999a). However, the *Drosophila* ortholog of WIF-1, *shifted*, appears to act as an inhibitor of the Hh pathway and not the Wingless pathway (Glise et al., 2005; Gorfinkiel et al., 2005). Cerberus, a head-inducer identified in *Xenopus*, appears to antagonise multiple signalling pathways: Nodal, BMP and Wnt (Piccolo et al., 1999). However, functional homologs have not yet been identified in other species.

Dickkopf proteins inhibit Wnt signalling by binding to the LRP6 receptor, which, through the action of Kremen, causes the endocytosis of the receptor, preventing it from accessing Wnt (Mao et al., 2002). Currently, no fly homologs of either Kremen or Dickkopf have been identified. However, mouse Dkk1 and Kremen2 can inhibit

Wingless signalling in both flies and tissue culture assays when overexpressed (Mao et al., 2002). In addition to Dickkopf proteins, the secreted inhibitor Wise has been shown to inhibit Wnt signalling by binding to the LRP6 receptor. Wise shares a binding site with XWnt8 on LRP6 and has been shown to block Wingless from accessing its receptor (Itasaki et al., 2003).

As none of these molecules have homologs in *Drosophila*, the role of secreted inhibitors in the regulation of Wingless signalling appears to be limited. However, the identification of Notum (also referred to as Wingful), an extracellular inhibitor of Wingless, suggests that secreted inhibitors do play a role in controlling Wingless signalling levels (Gerlitz and Basler, 2002; Giraldez et al., 2002). Notum is a secreted protein that contains homology to hydrolytic enzymes. In the absence of Notum, Wingless signalling is increased and ectopic expression of Notum results in a reduction in Wingless signalling. The mode of action of Notum is not currently clear, but the observation that it can cleave Dally-like, suggests a possible mechanism where upon cleavage of Dally-like by Notum, Wingless bound to Dally-like is shed from the cell and is consequently unavailable for signalling (Kreuger et al., 2004).

1.8.2 Regulation of Receptor Levels

Limiting the receptor levels present at the cell surface is an efficient method of preventing excess signalling. Wingless has been shown to regulate the expression of a number of its receptors, including members of the Frizzled family, Arrow and Dally-like. Dfz2 expression is repressed by Wingless signalling (Cadigan et al., 1998; Lecourtois et al., 2001; Muller et al., 1999) and Dfz3, a receptor speculated to act as an attenuator of Wingless signalling, shows increased expression upon Wingless signalling (Sato et al., 1999). Expression of Arrow also appears to be inhibited by Wingless signalling in both the embryonic epidermis and the wing disc (Wehrli et al., 2000) and Dally-like is repressed in response to Wingless signalling (Han et al., 2005). Repression of the Wingless signalling receptors in response to Wingless is likely to have an effect, not only on Wingless signalling levels, but also, as the receptors provide capturing activity, on the global distribution of Wingless protein. As the receptors provide much of the capturing activity, repression of receptors such as Dfz2, which have a high affinity for Wingless (Wu and Nusse, 2002), has been postulated to help shape the

morphogen gradient and allow movement of Wingless far from the source (Cadigan et al., 1998).

1.8.3 Wingless transport across epithelia

The mechanism of transport of secreted signalling molecules is of central importance to their ability to activate signalling at the appropriate levels in receiving cells. A number of different mechanisms of transport of Wingless have been proposed, the most prominent of these being passive diffusion and planar transcytosis (Figure 1.7). Two further possible mechanisms that have been proposed involve cytonemes, which are long filopodial processes that extend towards the signalling centre or argosomes, lipoprotein particles that could carry Wingless.

The two epithelia in *Drosophila* that are most amenable to the study of Wingless transport are the embryonic epidermis and the larval imaginal discs. As embryonic development occurs relatively rapidly and the distances over which Wingless is transported in the embryo are relatively short, much of the research has focused on the disc.

In the wing disc, it is unlikely that the Wingless gradient could be established by free diffusion as there is a large extracellular lumen around the wing disc epithelium and consequently, free Wingless would be likely to be lost in the extracellular space. The wing disc epithelium is also highly convoluted and a free diffusion mechanism would not follow the folds of the disc. It has therefore been suggested that Wingless might associate closely with the membrane of the transporting cells and be transported by a process of restricted diffusion (Vincent and Dubois, 2002) (Figure 1.7). Indeed, the discovery that Wingless in palmitoylated (Willert et al., 2003) suggests that it is likely to associate closely with membranes. Alternatively, the association of Wingless with proteoglycans could maintain its association with the membrane, while allowing cell-to-cell transfer. Wingless is believed to be secreted apically and form an extracellular basolateral gradient in the imaginal disc (Strigini and Cohen, 1999), it is not yet clear how Wingless in a diffusion model crosses the epithelial barrier from apical to basal.

Transcytosis of proteins depends on sequential rounds of endocytosis and recycling to the cell surface (Figure 1.7). If, during each cycle, a certain quantity of the ligand were targeted to lysosomes following endocytosis, then a gradient of Wingless protein would

be formed. Consequently, the rate of recycling and degradation would specify the slope of the gradient (Vincent and Dubois, 2002).

Evidence from the wing imaginal discs argues against a mechanism of planar transcytosis of Wingless. In this tissue, in the absence of *shibire*, the Wingless gradient forms normally and transport can occur through the mutant tissue (Strigini and Cohen, 2000), suggesting that in this tissue endocytosis is not required for Wingless transport. Furthermore, the Wingless gradient has been shown to form quickly (covering 50μm in 30 minutes (Strigini and Cohen, 2000)), and theoretical studies on gradient formation have suggested that this speed would be incompatible with transcytosis (Lander et al., 2002).

A variation on the theme of transcytosis was proposed by Greco et al., (2001). The researchers observed structures labelled with GFP-GPI (a glycosyl phosphatidyl inositol anchor fused to GFP) that travel at a rate comparable to that exhibited by Wingless. These structures were termed 'Argosomes' and were shown to colocalise with Wingless in endocytic structures (Greco et al., 2001). While originally thought to be membrane exovesicles, a recent study suggested that Argosomes could be comprised of lipoprotein particles (Panakova et al., 2005). Restricting lipid intake of developing larvae narrows the range of Wingless protein and signalling activity, suggesting a requirement for lipoproteins in Wingless transport. However, a reduction in lipid is likely to have multiple effects on the growth of the disc, so the contribution of lipoprotein particles to Wingless transport remains questionable.

A further model that was proposed invoked structures termed cytonemes, these are thin, actin-based projections that project towards the signalling centre and could allow receipt of signals (Ramirez-Weber and Kornberg, 1999). However, cytonemes have proved very difficult to observe *in vivo* and while projections can be observed in cultured imaginal disc cells, their contribution to the delivery of secreted signalling molecules *in vivo* is not yet confirmed.

Current evidence from the *Drosophila* embryo supports a model of planar transcytosis, embryos mutant for *shibire* (the fly homolog of dynamin) have a perturbed Wingless gradient and Wingless accumulates at the periphery of the producing cells (Bejsovec and Wieschaus, 1995; Moline et al., 1999). Interestingly, in the embryo, a membrane-tethered form of Wingless is still able to activate signals at a distance. A finding that suggested that the progeny of the Wingless secreting cells move away from the source

and, while they no longer produce Wingless, it is retained by the daughter cell and can consequently signal to the neighbouring cells (Pfeiffer et al., 2000).

Together, this evidence suggests that Wingless transport could occur by two different mechanisms in the embryo and the imaginal discs. This is likely to be due to the different requirements of the respective tissues. In the embryo, where the distance of transport is relatively short, retention of the ligand by the progeny of the producing cells, with a requirement for endocytosis is enough to reach the target cells. However, in the wing disc, when the distance of transport is greater, a mechanism of guided diffusion, possibly involving argosomes, would be more efficient in establishing the gradient.

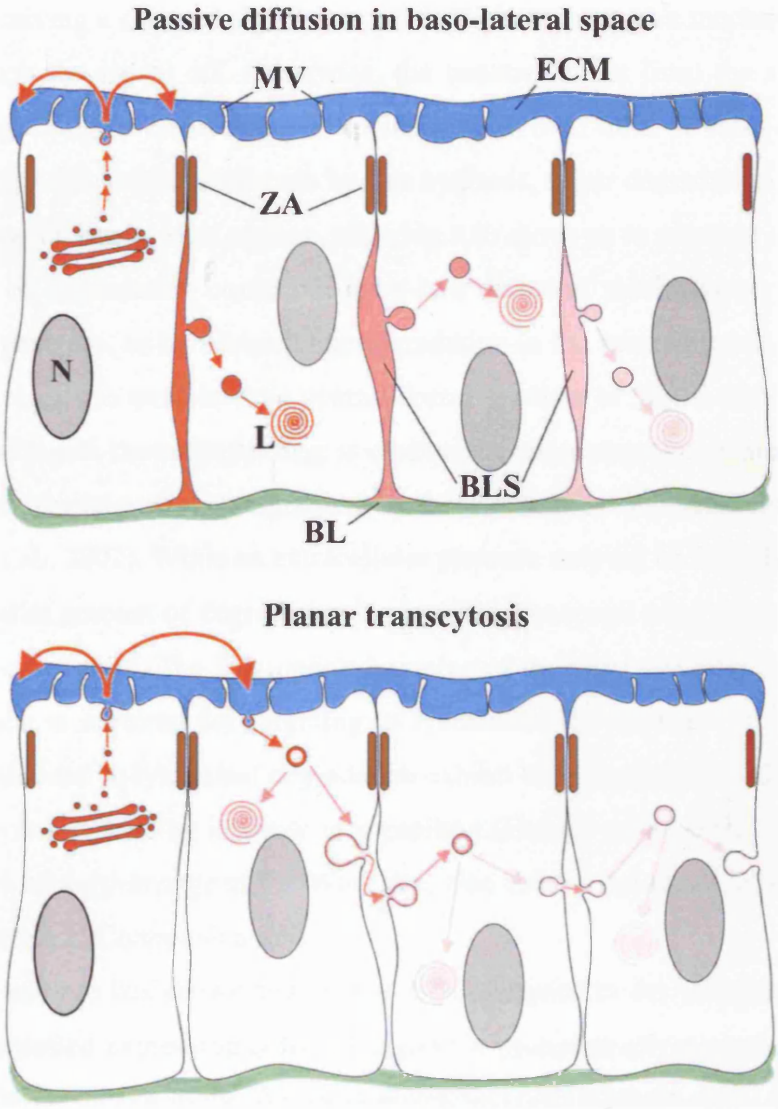


Figure 1.7 Two models of Wingless transport in the imaginal disc

Wing imaginal discs contain microvilli (MV), zonulae adherens (ZA) and a basal lamina (BL). Wingless is secreted apically and forms a basolateral gradient (Strigini and Cohen, 2000). However, it is not currently clear how Wingless crosses the epithelial barrier from apical to basal.

In the passive diffusion model, following secretion, Wingless freely diffuses in the baso-lateral space (BLS), and forms a gradient. Degradation of Wingless occurs in lysosomes (L).{Vincent, 2002 #150}

1.8.4 Wingless Degradation

If a cell is receiving a secreted signal in a gradient, there must be a mechanism in place that can switch the signal off. Otherwise, the constant input from the source would mean that signalling levels in the cell would increase over time. A cell could feasibly remove a secreted signalling molecule by two methods, either degradation of the ligand or by releasing the ligand after capture, allowing it to move on to adjacent cells.

The process of degradation could occur by two different mechanisms, either by an extracellular protease, or by intracellular degradation in the receiving cell. An example of the former exists to modulate the ventral-dorsal gradient of Sog, a secreted inhibitor of Dpp signalling in the embryo. Sog is cleaved by the extracellular metalloprotease Tolloid, which forms a converse gradient to that of Sog and mediates its degradation (Srinivasan et al., 2002). While an extracellular protease may act on Wingless, it is clear that a substantial amount of degradation occurs in a lysosomal compartment. Embryos mutant for *deep-orange* (The *Drosophila* homolog of the yeast vacuolar sorting protein Vps18p, which is required for targeting to lysosomes (Sevrioukov et al., 1999)) or chemically inhibited in lysosomal degradation exhibit both increased levels of Wingless protein and a corresponding increase in signalling (Dubois et al., 2001). Furthermore, mutant clones of *deep-orange* in the Wing disc also exhibit increased Wingless protein (L. Dubois Personal Communication).

Work in the embryo has shown that in the cells posterior to the Wingless expressing cells, (the Engrailed expressing cells), Wingless is preferentially degraded at a higher rate than in those anterior to the Wingless producing cells (Dubois et al., 2001) (Figure 1.3). In this study, a fusion protein of Wingless to horseradish peroxidase (HRP) was created. Due to the stability of the HRP component, it can still observed even after the Wingless moiety has been degraded in lysosomes. This allowed the investigators to observe where Wingless had been, even after lysosomal degradation. It was found that Wingless does reach the cells in the engrailed domain. However, here it is endocytosed and degraded at a higher rate than in the anterior cells. This mechanism is utilised to convert the symmetric Wingless gradient that is present in the embryo until stage 10, into an asymmetric gradient that is observed from stage 11.

These findings indicate that endocytosis and lysosomal degradation are of key importance in the regulation of Wingless distribution and activity. This observation

forms the basis of the work described in this thesis. Presumably, endocytosis and degradation of Wingless are mediated by the receptors. The aim of this work is to identify the mechanism by which this occurs. Receptor-mediated endocytosis and degradation is a commonly utilised method of regulating the distribution and activity of secreted signalling molecules, the following sections describe this process.

1.9 Endocytosis

Endocytosis is the mechanism by which cells take up extracellular molecules, particulate matter and portions of their own membrane into the cytoplasm. In general, endocytosis begins with deformation of the plasma membrane in a region where certain constituents (cargo) are sequestered. The invaginations then detach from the membrane, giving rise to intracellular vesicles where the cargo is directed to the appropriate cellular destination. Multiple endocytic pathways have been identified, which regulate the uptake of a diverse array of substances such as membrane proteins and lipids, pathogens such as bacteria and viruses, cell debris, extracellular fluid, nutrients and ligand-receptor complexes from the plasma membrane. The endocytic pathways can be divided into two broad categories, phagocytosis, the uptake of large particles and pinocytosis, the uptake of fluid and solutes (Conner and Schmid, 2003).

Phagocytosis is the process by which specialised phagocytic cells take up pathogens such as bacteria and yeast and also apoptotic cells. It is essential for host defence against infection and the clearance of cellular debris. Specific receptors on phagocytic cells recognise molecular markers on the target. This induces rearrangements in the actin cytoskeleton, which leads to the engulfment and uptake of the target (Aderem and Underhill, 1999). In *Drosophila*, macrophages primarily mediate phagocytosis and a number of phagocytic receptors, such as Croquemort and Draper, which mediate phagocytosis of apoptotic cells, have been identified (Franc et al., 1999; Manaka et al., 2004). However, more pertinent to this thesis is the process of pinocytosis, which occurs via a number of different pathways including clathrin-dependent endocytosis, macropinocytosis, caveolae, and clathrin and caveolin-independent mechanisms. The following section describes these processes.

1.9.1 Clathrin-dependent Endocytosis

The best understood mechanism of pinocytosis is the clathrin-dependent pathway which involves the uptake of coated vesicles from the plasma membrane and regulates the uptake of transmembrane receptors such as low-density lipoprotein receptors, the transferrin receptor and cell surface signalling receptors. Clathrin-mediated endocytosis involves the formation of coated pits, membrane invaginations surrounded by a coat of polymerised clathrin where cargo molecules are concentrated. Clathrin coated pits (CCPs) then detach from the membrane, giving rise to clathrin coated vesicles (CCVs) (Figure 1.8)

The formation of CCPs arises from the association of adaptor proteins with motifs in the cytoplasmic domains of receptors, phosphoinositides and membrane proteins such as Synaptotagmin, which may act as docking sites for the adaptor proteins. The adaptor proteins then recruit clathrin, which forms the structural basis of the coat, to the site of pit assembly. The best-characterised adaptor protein is AP-2, which is a multi-subunit complex and is a major component of clathrin coats. Adaptor proteins, in addition to there role in connecting cargo to the CCP, also promote the polymerisation of clathrin triskelia resulting in the formation of the clathrin coat, a function which leads to them also being referred to as assembly proteins.

Clathrin: Clathrin has a three-legged structure, termed a triskelion, comprised of three clathrin heavy chains of approximately 190kD, each with an associated light chain of approximately 25kD. The c-terminus of the clathrin heavy chain mediates trimerisation into triskelions and the n-terminus interacts with a number of clathrin accessory proteins, which are associated with CCPs. Electron microscopy of clathrin-coated vesicles indicates that polymerised clathrin surrounding CCVs is present in a basket-like structure comprised of hexagons and pentagons that surrounds the coated vesicle (Heuser, 1980). Under certain non-physiological conditions, clathrin polymerisation can spontaneously occur, resulting in the formation of basket-like structures of polymerised clathrin triskelia. However, the formation of clathrin baskets under physiological conditions requires assembly (adaptor) proteins (Pley and Parham, 1993).

1.9.1.1 Adaptor Proteins

AP-2: AP-2 is a heterotetrameric complex comprised of two large subunits of approximately 100kDa (α and β2 adaptins), a medium subunit of approximately 50kDa (μ2 adaptin) and a small subunit of approximately 25kDa (σ2 adaptin). The AP2 complex is comprised of a core domain consisting of the N-termini of the α and β2 subunits, the μ2 and the σ2 subunits and two appendages or 'ear' domains linked to the core by an extended flexible hinge region consisting of the C-termini of the α and β2 subunits (Collins et al., 2002; Kirchhausen, 1999; Kirchhausen et al., 1989). The hinge and appendage of the β2 subunit bind clathrin and stimulate clathrin polymerisation (Owen et al., 2000) (ter Haar et al., 2000). The α subunit binds phospholipids, phosphatidylinositol 4,5-bisphosphate (PtdIns[4,5]P_2) targeting the complex to the plasma membrane (Gaidarov and Keen, 1999). The appendage domains of both the α and β2 subunits bind accessory proteins including amphiphysin, Eps15, and epsin which are accessory proteins involved in clathrin-mediated endocytosis (Slepnev and De Camilli, 2000). Endocytic motifs in transmembrane proteins bind to the core domain of the complex. YXXϕ motifs bind the μ2 subunit (Ohno et al., 1995) and it has been suggested that dileucine motifs bind the β2 and the μ2 subunits (Rapoport et al., 1998; Rodionov and Bakke, 1998), however this finding has been questioned (Janvier et al., 2003).

AP-2 has been considered to be the master controller of CCV formation, however in yeast, depletion of all the AP components does not effect clathrin-mediated endocytosis (Yeung et al., 1999) and furthermore, EGF receptor internalisation is unaffected in HeLaB cells expressing a mutant form of the μ2 subunit of AP-2. This suggests that to some extent AP-2 may be dispensable for CDE (Motley et al., 2003).
In addition to AP-2 and clathrin, CCVs have a number of other associated proteins that play a role as adaptor proteins, recruiting transmembrane receptors to CCPs, facilitating clathrin assembly and fission from the plasma membrane. Accessory proteins are generally characterised by binding sites for components of the clathrin coat including clathrin, AP-2, other accessory proteins and also phospholipids.

1.9.1.2 Other adaptors for clathrin dependent endocytosis

β-Arrestins: β-Arrestins act as adaptor proteins in the endocytosis of seven-pass transmembrane, G-protein coupled receptors (GPCRs). β-Arrestins are monomeric proteins of approximately 45kDa which bind phosphorylated GPCRs and mediate endocytosis via interactions with clathrin (Goodman et al., 1996), the β2 adaptin subunit of AP-2 (Laporte et al., 2002) and PtdIns[4,5]P_2 (Gaidarov et al., 1999).

Disabled-2: Disabled-2 is a member of a group of phosphotyrosine binding (PTB) domain proteins have been implicated in clathrin-dependent endocytosis on receptors containing the FxNPxY motif. The PTB domain of Disabled-2 binds the FxNPxY motif, present on the LDL receptor, and interacts with clathrin, AP-2 and PtdIns(4,5)P_2 (Mishra et al., 2002; Morris and Cooper, 2001). Furthermore, Disabled-2 can stimulate clathrin polymerisation.

Epsins have been proposed to act as adaptor proteins for clathrin-dependent endocytosis, Epsins can bind clathrin, AP-2 and PtdIns[4,5]P_2, and have a ubiquitin-binding domain that has been suggested to function as a cargo-binding domain for ubiquitinated receptors (Wendland, 2002). In addition to their role in clathrin-mediated endocytosis, epsins are also are involved in clathrin-independent endocytosis (Aguilar and Wendland, 2005) (Chen and De Camilli, 2005; Sigismund et al., 2005). Upon binding to PtdIns[4,5]P_2 Epsins may induce the curvature of the membrane required for the formation of the CCP (Ford et al., 2002).

AP-180/CALM: The neuronally expressed AP-180 and its more widely expressed relative CALM (clathrin assembly myeloid leukaemia) are monomeric proteins that bind AP-2, clathrin and PtdIns[4,5]P_2. AP180 does not bind cargo molecules but stimulates clathrin polymerisation and may regulate the size of vesicle formation.

1.9.1.3 Fission of clathrin-coated pits

Fission of CCPs is primarily mediated the GTPase dynamin, which was initially implicated in endocytosis in *Drosophila.* At restrictive temperature, temperature-sensitive *shibire* mutants arrest synaptic vesicle endocytosis and accumulate CCPs at the plasma membrane (Koenig and Ikeda, 1989; Kosaka and Ikeda, 1983). Subsequently *shibire* was identified to encode the *Drosophila* homolog of Dynamin (Chen et al., 1991) and has been identified to be involved in both clathrin-dependent and independent endocytic pathways (Hinshaw, 2000). The precise mechanism of dynamin-mediated vesicle budding is not known, but some evidence suggests that dynamin could act as a mechano-chemical enzyme that physically drives budding by constriction or stretching of dynamin rings or coils located at the neck of CCPs, upon transition of dynamin from the GTP to the GDP bound state. Alternatively, dynamin may act as a regulatory GTPase, mediating interactions with downstream components that are required for clathrin-coated vesicle formation (Sever et al., 2000). After internalisation, the clathrin coat dissembles in a process mediated by Hsc70 and auxilin in an ATP-dependent reaction.

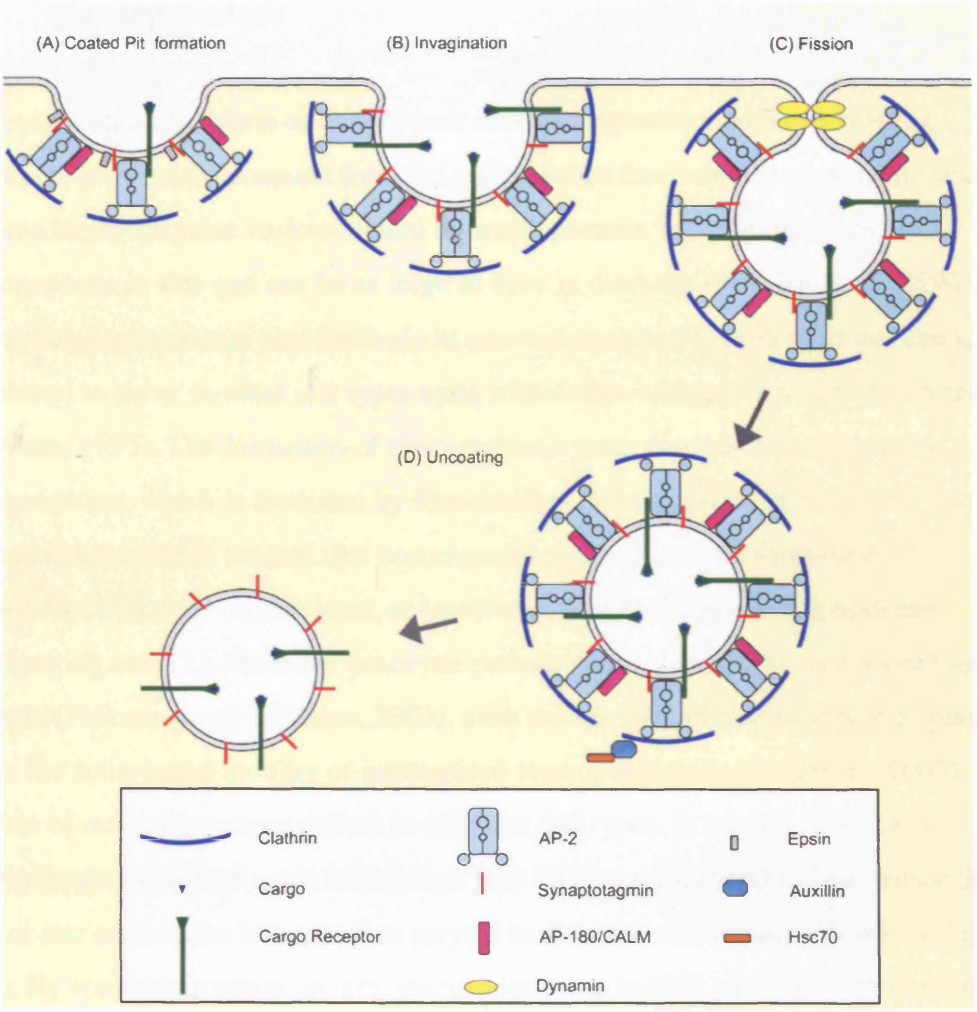


Figure 1.8 General scheme of clathrin-dependent endocytosis

For clarity, only some of the components are shown. At the site of coated pit formation AP-2 associates with synaptotagmin, phosphoinositides and tyrosine based motifs present in the cytoplasmic tails of cargo receptors. Epsin may induce membrane curvature upon insertion into the inner leaflet and along with AP-2 and AP180 mediate the assembly of the clathrin cage (A). AP-2 and other adaptor proteins recruit cargo receptors to the coated pit (B). Dynamin is recruited to the neck of deeply invaginated coated pits and mediates fission from the plasma membrane resulting in the formation of free-coated vesicles (C). After internalisation Auxilin and Hsc70 mediate uncoating of the vesicle (D).

1.9.2 Macropinocytosis

Macropinocytosis is a form of endocytosis that accompanies membrane ruffling. Membrane protrusions emanate from the cell and then fuse with the plasma membrane to create large, irregular vesicles called macropinosomes. Macropinosomes are heterogeneous in size and can be as large as 6μm in diameter (Hewlett et al., 1994). Macropinocytosis occurs constitutively in macrophages and tumour cells but can also be induced to occur in other cell types upon stimulation with growth factors (Swanson and Watts, 1995). The formation of the membrane protrusions is driven by actin polymerisation, which is mediated by Rho-family GTPases (West et al., 2000). Little is know about the fusion process that accompanies macropinosome formation. Presumably, this must be regulated, at least to prevent fusion occurring between neighbouring cells. Unlike other pinocytic pathways, macropinocytosis does not require dynamin (Pelkmans and Helenius, 2003), even though dynamin does appear to play a role in the actin-based motility of internalised macropinosomes (Orth et al., 2002).
The fate of macropinosomes differs in different cell types. In human A341 cells, macropinosomes formed upon stimulation with EGF are not found to fuse with either early or late endosomes but appear to recycle back to the cell surface (Hewlett et al., 1994). By contrast, in macrophages, macropinosomes acidify and fuse with lysosomes, resulting in the degradation of the contents (Racoosin and Swanson, 1993).
Macropinocytosis can play diverse roles. In dendritic cells, it leads to both the MHC class I and class II antigen presentation pathways (Watts, 1997). Macropinocytosis induced in response to growth factors may be required to enhance the uptake of nutrients required for cell growth and may stimulate motility (Ridley, 2001). Finally, macropinocytosis has been exploited by a number of pathogens such as Legionella (Watarai et al., 2001) and HIV for cell entry. After exposure to HIV-1, HIV particles are observed in macropinosomes. Most of the virons are subsequently degraded, but a fraction of the virons may escape degradation and be able to lead to productive infection of macrophages (Marechal et al., 2001).

1.9.3 Caveolae

Caveolae are small, flask shaped imaginations present on the membrane of many cell types and are particularly abundant on endothelial cells. Caveolae are associated with lipid rafts, which are detergent-resistant, low-density membrane fractions that are rich in cholesterol and glycosphingolipids. Caveolae are characterised by the presence of caveolins, which are a family of integral membrane, cholesterol-binding proteins (Rothberg et al., 1992). Caveolins clearly play an important role in the generation or maintenance of caveolae as caveolin-1 knockout mice are devoid of caveolae. Moreover, expression of caveolin in cells that lack caveolae is sufficient for their formation (Drab et al., 2001; Fra et al., 1995). Caveolae are located on microdomains of the plasma membrane that are enriched in cholesterol, glycosphingolipids and lipid-anchored membrane proteins. The appearance of caveolae as plasma membrane invaginations and the localisation of dynamin to the necks of caveolae suggest that they may bud from the plasma membrane, however the role of caveolae in endocytosis has been a source of much debate.

Somewhat surprisingly caveolin-1 knockout mice are viable. Still, a number of mild phenotypes have been identified that suggest functional roles for caveolae. Lung endothelial cells of caveolin-1 null mice exhibit a hyperproliferative response, suggesting a role in the regulation of signalling cascades. This observation is consistent with the observation that caveolae are associated with a number of membrane receptors, signalling molecules and membrane transporters (Ceresa and Schmid, 2000; Drab et al., 2001; Razani et al., 2002). Also, mouse embryonic fibroblasts isolated from Caveolin-1 knock-out mice exhibit a decrease in albumin endocytosis, consistent with the previously reported role of caveolae in mediating albumin uptake (Ghitescu et al., 1986; Razani et al., 2002).

Studies using GFP-tagged caveolin-1 suggest that caveolae are highly immobile at the plasma membrane and only very few caveolin-1 positive vesicles are internalised. Suggesting that caveolae do not contribute to constitutive endocytosis (Thomsen et al., 2002). However, internalisation of caveolae can occur upon phosphorylation of caveolae components and can be stimulated by phosphatase inhibitors or by cargo that bind to receptors present in caveolae (Stang et al., 1997) (Parton et al., 1994). Therefore suggesting that caveolae internalisation may be a highly regulated process.

An example of caveolar endocytosis is the uptake of SV40. SV40 virons are localised to caveolae (Stang et al., 1997). Using a GFP-tagged caveolin-1, SV40 has been shown to be co-internalised with caveolin-1 into endosomal structures termed caveosomes (Pelkmans et al., 2001). Caveosomes represent non-classical endosomal structures that do not colocalise with markers for CDE such as transferrin and are of neutral pH. After internalisation into caveosomes, SV40 is then sorted to caveolin-1 negative membrane vesicles, where it is transported to the smooth ER in a microtubule dependent manner. Internalisation of SV40 can also occur in the absence of caveolae (Damm et al., 2005), suggesting that it can utilise multiple pathways of uptake. Internalisation of caveolae is mediated by the GTPase dynamin, which localises to the necks of caveolae, suggesting a similar method of fission to that suggested for clathrin-mediated endocytosis (Henley et al., 1998; Oh et al., 1998).

While these processes suggest roles for caveolae in endocytosis, some issues still remain. For example, the relatively mild phenotypes observed in caveolin-1 knock out mice suggest that compensatory measures can be applied that can overcome absence of caveolae. Overexpression of caveolin-1 GFP, leads to a reduction of internalisation of gp60, a receptor for albumin that is localised to caveolae in endothelial cells (Minshall et al., 2000), and a reduction in the uptake of cholera toxin B subunit (Le and Nabi, 2003) Suggesting that caveolin-1 could act to stabilise membrane invaginations.

1.9.4 Clathrin and caveolin independent endocytosis

In addition to caveolin mediated endocytosis of lipid rafts domains, it has become clear that endocytosis of rafts can occur in the absence of caveolin via a number of different mechanisms. However as yet, these processes are relatively poorly understood and have mainly been described in negative terms depending on their independence from clathrin, caveolin or dynamin.

Endocytosis via lipid rafts has been suggested to mediate uptake of interleukin-2 (IL-2) receptors in lymphocytes (Lamaze et al., 2001). Perturbation of clathrin-dependent endocytosis in fibroblasts blocks Transferrin receptor uptake, yet has no effect on the uptake of IL-2 receptors. Furthermore, in lymphocytes, which are devoid of caveolae, IL-2 receptor associates with detergent-resistant membranes both before and after internalisation (Lamaze et al., 2001). This suggests that IL-2 receptor internalisation is

coupled to its association with lipid rafts and occurs independent of caveolae. In common with its role in CDE and internalisation of caveolae, internalisation of IL-2 is dependent on dynamin (Lamaze et al., 2001), suggesting a common mechanism for membrane fission.

Dynamin does not appear to be required for all internalisation events from rafts. GPI-anchored proteins associate with rafts, however, internalisation via clathrin and caveolin independent mechanisms is unaffected by the presence of dominant negative dynamin (Sabharanjak et al., 2002). Indicating that multiple pathways of endocytosis occur from lipid rafts, and that the mechanism of internalisation depends on the cargo.

1.10 Endocytosis and Signalling

The role of endocytosis in cell signalling has historically been viewed as a mechanism by which signalling responses are downregulated. Internalisation of receptors from the cell surface reduces the ligand capturing ability of the cell and internalisation of ligand-receptor complexes, followed by targeting to lysosomes, can act to switch off signalling. However, it has recently become clear that endocytosis can play a positive role in m mediating signalling. This can take a number of forms. Firstly signal transduction can continue from endocytic compartments
(TGFB and SARA). Secondly, internalisation and sequestration of inhibitory receptors can lead to the release of signal inhibition (Hh), Thirdly, endocytosis can play a role in the dispersal of ligands which may contribute to the formation of morphogen gradients (Dpp). I will now discuss key examples for each of these cases and follow with a discussion of the possible role of endocytosis in regulating Wingless signalling.

1.10.1 TGFβ

TGFβs (transforming growth factor β) are a large family of growth factors that are involved in the regulation of multiple cellular processes in developing animals including differentiation and apoptosis (Massague, 1998; Whitman, 1998). Signal transduction occurs after binding of TGFβ to its type II receptor. This forms a complex with a Type I receptor, which is consequently phosphorylated. The activated Type I

receptor then phosphorylates Smad proteins, which transduce the signal and translocate to the nucleus (Massague, 1998).

Phosphorylation of Smad2 requires SARA (Smad Anchor for Receptor Activation), which recruits Smad2 to the receptor (Tsukazaki et al., 1998). SARA can interact with Smad2, the TGFB receptor complex and contains a PtdIns3P-binding FYVE zinc-finger domain, which is required for its localisation to early sorting endosomes (Tsukazaki et al., 1998). Deletion of the FYVE domain in SARA leads to its mislocalisation to the cytoplasm. This consequently leads to the mislocalisation of Smad2 and the inhibition of TGFB signalling (Tsukazaki et al., 1998). Therefore, recruitment of Smad2 to early endosomes by SARA is required for the activation of signalling. However, in the absence of endocytosis, the trimeric receptor-SARA-Smad2 complex can form at the plasma membrane indicating that recruitment of Smad2 can occur prior to endocytosis (Penheiter et al., 2002). Importantly however, phosphorylation of Smad2 and the consequent activation of signalling are dependent on the endocytosis of this complex, demonstrating the requirement for endocytosis in the activation of signalling (Penheiter et al., 2002).

TGFβ receptor endocytosis can occur through both clathrin-dependent and independent mechanisms (Di Guglielmo et al., 2003). Endocytosis through clathrin-coated pits leads to the propagation of signals, whereas endocytosis in caveolae leads to the association of the TGFβ receptor with Smad7 and Smurf proteins (Di Guglielmo et al., 2003). Smurf proteins are E3 ubiquitin ligases that regulate the TGFβ signalling pathway by modulating degradation of both the receptors and the Smads (Ebisawa et al., 2001; Kavsak et al., 2000) (Zhu et al., 1999). In mammalian cells, Smurf proteins are recruited to the activated receptor complex by Smad7. This leads to ubiquitination of Smad7 (Kavsak et al., 2000) and the Type I receptor (Ebisawa et al., 2001), leading to their degradation and consequently a reduction in signalling activity (Ebisawa et al., 2001). The two pathways of TGFβ receptor endocytosis therefore can act to modulate signalling levels, clathrin-dependent endocytosis into early endosomes activates signalling through Smad proteins, whereas internalisation through caveolae leads to the ubiquitination and degradation of the receptor and the downregulation of signalling (Di Guglielmo et al., 2003).

1.10.2 EGF

One of the best-characterised examples of endocytosis and degradation regulating signalling molecules exists for the Epidermal Growth Factor (EGF) pathway. EGFs are secreted proteins that stimulate cell growth and division by binding to and activating the Epidermal Growth Factor Receptor (EGFR) (Jorissen et al., 2003).

Upon binding to EGFR, EGF is internalised by clathrin-dependent endocytosis and subsequently targeted to lysosomes (Futter et al., 1996; Haigler et al., 1979). Ubiquitination of the receptor, which is required for degradation of the ligand, is contingent on the tyrosine phosphorylation that accompanies receptor activation (Shtiegman and Yarden, 2003). This ensures that degradation only occur after signalling has been activated. Prevention of EGFR endocytosis in NIH 3T3 cells causes increased EGF-dependent cell proliferation (Wells et al., 1990), indicating that endocytosis plays a role in downregulating the response to the secreted signal. However, blocking endocytosis does not result in activation of all of the EGF-dependent responses of the cell (Vieira et al., 1996). This suggests that endocytosis is required for activation of certain responses and can be a means used by cells to modulate response to the signal.

The role of endocytosis in the downregulation of EGFR signalling has also been observed in *Drosophila*. Mutants for *hrs*, the homolog of the yeast vacuolar sorting protein Vps27p, which is required for endocytosis and targeting to multi-vesicular bodies (MVBs), fail to downregulate activated EGFR, resulting in increased signalling (Lloyd et al., 2002).

1.10.3 Hedgehog

The Hh signalling pathway is activated upon binding of Hh to the trans-membrane protein Patched (Ptc). This relieves the inhibition of Ptc on another transmembrane protein Smoothened (Smo), which activates the downstream pathway (Murone et al., 1999). Ptc ostensively has two functions in Hh signalling, the sequestration of Hh and the inhibition of Smo, both of which result in reduced Hh signalling.

The sequestration of Hh by Ptc occurs in endosomes, Ptc1 has been shown to be involved in the receptor-mediated endocytosis of Hh proteins in both vertebrates

(Incardona et al., 2000) and *Drosophila* wing discs (Torroja et al., 2004). However, the requirement for Ptc internalisation in Hh signalling has been a subject of some debate. Upon addition of ligand, Ptc and Smo and readily endocytosed together into late endosomes (Incardona et al., 2002), after this event, Smo is segregated from the Ptc/Hh complex (which is targeted to lysosomes for degradation) suggesting a possible mechanism by which Smo inhibition is released (Incardona et al., 2002).
However, evidence from *Drosophila* suggests that Ptc endocytosis is not essential for Hh signalling (Torroja et al., 2004). Indeed, the findings of Torroja el al (2004) suggest that the two functions of Ptc (sequestering Hh in endosomes and its consequent targeting to lysosomes and the inhibition of Smo) are separate and do not depend on the other. Whether Ptc endocytosis is the mechanism that enables Smo to activate signalling or not, endocytosis and degradation are clearly important in the regulation of Hh gradient formation, blocking either of these processes causes an increase in the levels of Hh (and Ptc) (Incardona et al., 2002; Torroja et al., 2004).

1.10.4 Wnt/Frizzled endocytosis

Frizzled proteins are similar in structure to GPCRs and activate Wnt signalling in a G-protein dependent manner (Katanaev et al., 2005). These similarities suggest that their endocytosis may be regulated in a similar manner. GPCRs are endocytosed by clathrin-dependent endocytosis. When GPCRs are activated, they are phosphorylated by G-protein-coupled receptor kinases. β-Arrestins can then bind activated GPCRs and prevent G-proteins and receptors interacting further. β -Arrestins interact with clathrin and AP-2 to mediate endocytosis of the receptor in clathrin-coated vesicles (Luttrell and Lefkowitz, 2002). A recent study found that indeed, Frizzleds are endocytosed in a similar manner and that β-Arrestin 2 mediates the endocytosis of mammalian Frizzled 4. A Fz4-GFP fusion protein expressed in human embryonic kidney cells is present mainly at the cell surface. However, upon stimulation by Wnt5a and an activator of Protein Kinase C, Fz4-GFP is observed in intracellular vesicles (Chen et al., 2003). Inhibition of clathrin-mediated endocytosis blocks Fz4 internalisation and RNAi against β-Arrestin 2 blocks Fz4 internalisation, indicating that β-Arrestin 2 plays a key role in clathrin-dependent endocytosis of Fz4. β-Arrestin-mediated endocytosis of Fz4 requires Dsh, which recruits β-Arrestin 2 to Fz4, demonstrating and link between downstream

signalling components and receptor endocytosis (Chen et al., 2003). Wnt5a stimulates this process, however, it is not yet known whether Wnt5a is internalised with Fz4 and whether this plays a role in signal downregulation. Work in this thesis investigates the role of Frizzled proteins in the endocytosis of Wingless.

1.11 How could wingless degradation be regulated by the receptors?

Degradation of secreted ligands can most simply be broken up into three steps: the capture of the ligand by the cell, endocytosis and finally targeting to lysosomes for degradation (Figure 1.9). This thesis will investigate how, in the case of Wingless, these processes are regulated.

In *Drosophila*, Dfz2 has the highest affinity for Wingless (Wu and Nusse, 2002) and Arrow can also contribute to binding (Cong et al., 2004; Tamai et al., 2000). However, it is not known how endocytosis and lysosomal degradation of Wingless are mediated. Dsh has been implicated in the endocytosis of Frizzled proteins (Chen et al., 2003) (see section 1.10.4), however, whether this leads to internalisation of Wnts is not yet known.

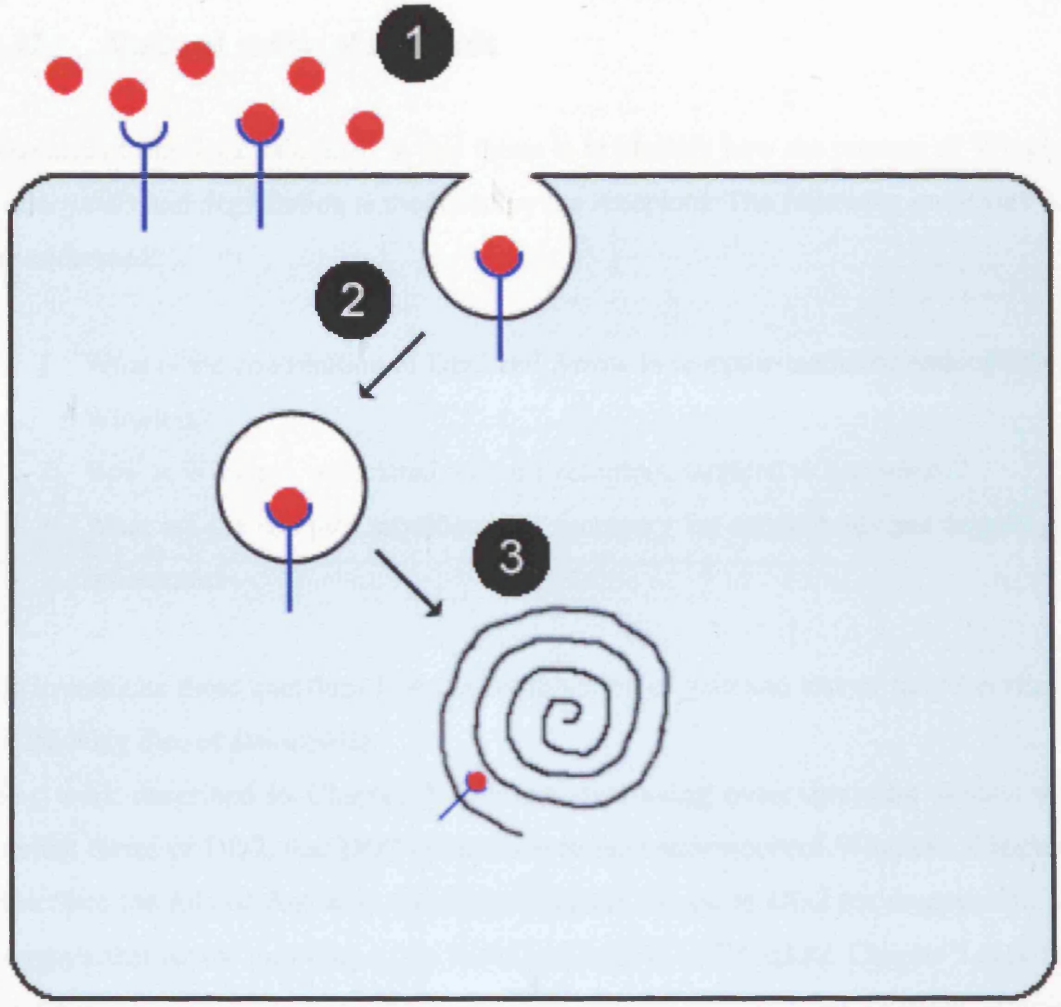


Figure 1.9 Three steps to ligand degradation

Ligand degradation in lysosomes is the outcome of three processes.

1. Capture by the receptor at the cell surface
2. Endocytosis
3. Targeting to lysosomes

1.12 Aims and outline of this thesis

The aim of the work described in this thesis is to identify how the process of Wingless endocytosis and degradation is mediated by the receptors. The following questions will be addressed:

1. What is the contribution of Dfz2 and Arrow to receptor-mediated endocytosis of Wingless?
2. How is Wingless, associated with the receptors, targeted to lysosomes?
3. What are the receptor modifications necessary for endocytosis and targeting to lysosomes?

To investigate these questions I used a combination of gain and loss of function studies in the wing disc of *Drosophila*.

The work described in Chapter 3 demonstrates, using overexpression studies with mutant forms of Dfz2, that Dfz2 contributes to the endocytosis of Wingless. Chapter 4 describes the role of Arrow in targeting Wingless bound to Dfz2 for degradation and suggests that Arrow may play a role in the endocytosis of Wingless. Chapter 5 describes preliminary work implicating Arrow phosphorylation as a key step towards Wingless degradation.

CHAPTER 2 – MATERIALS AND METHODS

2 CHAPTER 2 - MATERIALS AND METHODS

2.1 Fly Manipulations

2.1.1 Fly stocks

For the overexpression experiments the following stocks were used:
y w, dpp-gal4/TM6B; *apterous-gal4/GlaBc*; *y hsflp, wg [1-en-11]/CyO;* dpp-*gal4 UAS-Fz-2-FLAG/TM6B* (generated for this study); *UAS-arrow-HA* (generated for this study); *UAS-Dfz2-FLAG/CyO* (L. Dubois); *UAS-Dfz2-FLAG/TM3Sb* (L. Dubois); *UAS-Dfz2AV-FLAG/CyO* (L. Dubois); *UAS-fz2-GPI* (M. Cadigan); *w, UAS-armadillo [S10]* (M. Peifer); *UAS-ArrowΔC//CyO* (generated for this study); *UAS-dally-like* (S. Cohen); *UAS-dsh/TM6B*; *UAS-arrow-PPAP* (generated for this study); *UAS-arrowΔLysine* (generated for this study).

For the loss of function experiments the following stocks were used:
hs-flp, arr [2] pwn FRT42D/GlaBc (G. Struhl); *y w hsflp, FRT42D hs-GFP; FRT42D pcna/CyO-GFP, lama-gal4 UAS-flp* (I. Salecker); *UAS-CD8GFP hs-flp, FRT42D tubulin-gal80, tubulin-gal4* (S. Cohen); *dsh [3] FRT19A/FM7a* (Bloomington); *hs-flp, Sp/CyO, fz[H51] Dfz2[C1] ri FRT2A/TM3Sb* (G. Struhl); *hsflp, Ubi-GFP FRT2A/TM3Sb*; *Ubi-GFP FRT19A, lama-gal4/TM6B* (I. Salecker); *UAS-Arrow-HA; arr[2]/Gla Bc* (generated for this study); *w, arr[1] arm-gal4/GlaBc* (generated for this study); *w, arr[2] UAS-arrowΔC/CyO* (generated for this study).

2.1.2 Fly crosses and stock maintenance

Unless otherwise stated, all stocks were maintained at 18^{0}C and crosses were carried out at 25^{0}C in plastic vials containing standard organic media supplemented with dried yeast. In most crosses around 15 males and 15 females were used and progeny of the appropriate genotype were selected against dominant markers on balancer chromosomes.

2.1.3 Immunoflourescence in wing imaginal discs

2.1.3.1 Total staining

Wandering third instar larvae of the appropriate genotype were collected and dissected in PBS. The larvae were torn in half and the head inverted. Tissue surrounding the wing disc was removed and the dissected larval head placed in ice-cold PBS. Larval heads were then fixed in 4% paraformalydehyde in PBS for 20 minutes while rocking. After fixation, discs were washed 3-4 times in PBS. Blocking was carried out by washing three times for 15 minutes in PBX2' (PBSA + 0.05% Triton X-100) followed by 3 washes in BBX250' (PBSA, 0.05% Triton X-100, 250mM NaCl). Discs were then incubated in primary antibody in BBX (PBS + 0.05% Triton X-100 + BSA 1mg/ml) at 4^{0}C overnight. Discs were then washed twice for 20 minutes in BBX and twice for 30 minutes in BBX supplemented with 4% Fetal Calf Serum. Discs were then incubated in secondary antibodies for 1-2 hours at room temperature then washed for 4 x 15 minutes in PBX2' and 3 x 15 minutes in PBS. The discs were then removed from the larval heads and mounted in Vectashield with DAPI (Vector Laboratories). For each experiment 10-15 imaginal discs were analysed.

Imaging of samples was carried out on a Biorad Radiance 2100 laser scanning confocal microscope using Radiance 2100 software. Except in the cases of the Z-sections in

Figures 3.2, 3.4, 3.6, 3.11, 4.1, 5.1 and 5.4 where imaging was carried out on a Leica TCS SP laser scanning confocal microscope using Leica TCS software. Images were processed using Adobe Photoshop and assembled using Adobe Illustrator. In all images single confocal sections taken in the basolateral domain of the wing disc approximately 10μm below the apical surface. The thickness of the sections is ~285.8nm. Scale bars represent 10μm.

2.1.3.2 Extracellular staining

Extracellular staining was carried using a protocol adapted from Strigini and Cohen (2000). Wandering third instar larvae of the appropriate genotype were collected and dissected in ice-cold S2 medium (Sigma). Dissected larval heads were incubated in primary antibody in PBSA on ice for 1 hour and then washed twice in ice cold PBSA. Primary antibodies were used at three times the normal concentration. Discs were fixed for 5 minutes in 4% paraformaldehyde on ice followed by 20 minutes at room temperature. Blocking and secondary antibody labelling was carried out as for the total staining.

2.1.3.3 Antibodies

Primary antibodies used: mouse anti-Wingless 4D4 (DSHB, 1/10000), mouse M2 anti-FLAG (Sigma, 1/15000), rabbit anti-FLAG (Abcam, 1/3000), mouse anti-HA 1.1 (Babco, 1/3000), Alexa-488 mouse anti-HA 1.1 (Covance, 1/500), mouse anti- Myc (Roche, 1/600), rabbit anti-Myc (Santa Cruz, 1/500), rabbit anti-GFP (Abcam, 1/2500), rabbit anti-β–galactosidase (Cappel, 1/12000), rabbit anti-Arrow (E. Piddini 1/5000), rabbit anti-Dfz-2 (E. Piddini, 1/5000), rabbit anti-HA (Santa Cruz 1/400), Alexa488 chicken anti-FLAG (ICL, 1/500)

Secondary antibodies used: Alexa488 goat anti-rabbit, Alexa488 goat anti-mouse, Alexa594 goat anti-mouse, Alexa594 goat anti-rabbit (all Molecular Probes, 1/200), Cy5 goat-anti rabbit (Jackson1/200), Cy5 goat-anti mouse (Jackson1/200).

Dextran labelling

Method adapted from Entchev et al., (2000). Third instar larval discs were dissected in S2 medium and incubated in 0.5mM Texas-red dextran (lysine fixable, MW3000, Molecular Probes) at room temperature for 10 minutes. Then washed five times for 2 minutes with S2 medium on ice. Discs were then incubated in S2 medium for 20 minutes at room temperature, then fixed and stained as previously described.

2.1.4 Induction of mutant clones

For clones made using *hs flp*, flies of the appropriate genotype were allowed to lay in vials containing fly food overnight. Flies were then removed and the vials incubated at 25^{0}C for 48 hours. Larvae were heat shocked at 37^{0}C for 60 minutes to activate the flipase. Vials were incubated at 25^{0}C until the larvae reached third instar when they were dissected and stained as previously described. For clones made using *lama-gal4 UAS-flp*, flies were allowed to lay overnight it food vials. The parents were removed and the vials were incubated at 25^{0}C until the progeny reached third instar. Mutant clones of *arrow* and *Dfz1* and *Dfz2* were confirmed by the loss of Distal-less staining inside the clones (personal communication with E. Piddini)

2.1.5 Microinjection of Drosophila embryos

yw flies were allowed to lay on grape juice agar plates and embryos collected at one-hour intervals. Embryos were washed in PT (PBS, 0.1% Triton X-100) and

dechorionated in 50% bleach for 2 minutes. Dechorionated embryos were collected, aligned and glued to a coverslip. Embryos were then dried by placing the slide in a container of silica gel for 3 minutes and then overlaid with Voltalef 10S oil to prevent further desiccation. The DNA injection mix contained 4µl donor DNA, 1µl Turbo (Δ2-3 transposase), 4µl dH_2O, 1µl PBS. Uninjected or cellularised embryos were killed and injected embryos were kept in a humid container at 25°C for 24 hours. Hatched larvae were collected and placed in fly food vials and incubated at 25°C. Emerged flies were collected and crossed individually to *yw* flies. The progeny of this cross were screened for the presence of the w+ transgene and transformants collected.

2.1.6 Wing preparations

One to two day-old flies were collected and stored in methanol for 1 day. The wings were dissected in methanol, mounted individually in Euparal (Agar Scientific) and incubated overnight at 65°C. Wings were analysed and photographed on a Zeiss Axioplan 2 microscope.

2.1.7 *in situ* hybridisation

in situ hybridisation was carried out according to a protocol received from Cyrille Alexandre (NIMR, London). Larvae of the appropriate genotype were dissected in PBS and fixed in 3.8% formaldehyde. Discs were washed in PTW (PBS + 0.1% Tween-20) then rinsed 2-3 times in a 1:1 solution of PTW:hybridisation buffer (see below), followed by incubation for 2 hours in hybridisation buffer at 55°C. During the prehybridisation, the wingless probe (O. Marchand, NIMR, London) was heated to 80°C for 6 minutes. After prehybridisation, 600µl of fresh hybridisation buffer at 55°C was added to the discs and to this, the probe was added in 60µl of hybridisation buffer. Hybridisation took place overnight at 55°C.

The following day the discs were washed in hybridisation buffer at 55^{0}C for 20 minutes, followed by a 20 minute wash in 1:1 PTW: hybridisation buffer and 5-6 washes in PTW at room temperature.

Following the washes, discs were incubated in Anti-dioxygenin (Roche, 1/2000) in PTW for 1 hour at room temperature followed by 4 washes of 20 minutes in PTW. Discs were then rinsed twice in staining buffer (see below), followed by the addition of 1ml of staining buffer containing 4.5μl NBT and 3.5μl BCIP. The reaction was allowed to develop in the dark and stopped by multiple washes with PTW. Antibody staining was then carried out as previously described.

Hybridisation buffer: 50% deionised formamide, 5x SSC, 0.1µg/µl tRNA, 2.5mg/ml heparin, 0.1% Tween 20 in dH_2O.

Staining Buffer: 0.1M NaCl, 0.05M $MgCl_2$, 0.1M TrisHCl pH 9.5, 0.01% Tween 20 in dH_2O.

2.2 Molecular biology

2.2.1 General techniques

For preparation of DNA, the Qiaspin Miniprep kit (Qiagen) and the Qiafilter Plasmid MaxiPrep (Qiagen) kit were used according to the manufacturers instructions. Bacterial transformation, restriction digests, Agarose gel electrophoresis and PCR were carried out according to standard protocols (Sambrook and Russell, 2000).

2.2.2 Arrow constructs

Arrow-HA

C-terminal HA-tagged Arrow was generated by inserting DNA encoding a HA tag and a glycine linker into the *SnaBI* site of BSKS-Arrow (Steve DiNardo, University of Pennsylvania) to create BSKS-Arrow-HA.

The oligonucleotides used were:

Forward:

5'GGCTAGCGGAGGTGGCGGCGGAGGTGGAGGTGGCGGTTATCCTTATGACGTGCCTGA CTATGCGTAA

Reverse:

5'TTACGCATAGTCAGGCACGTCATAAGGATAACCGCCACCTCCACCTCCGCCGCCACC TCCGCTAGCC

BSKS-Arrow-HA was cut with *Asp718*, treated with the Klenow fragment of DNA polymerase I and then cut with *XbaI* to excise the Arrow-HA fragment.
pUAST was cut with *EcoRI*, treated with the Klenow fragment of DNA polymerase I and then cut with *XbaI* to create compatible ends for the Arrow-HA fragment. The two fragments were ligated and sequencing was used to verify its identity (Oswel Research).

ArrowΔC

To generate ArrowΔC, BSKS-Arrow was digested with *MluI* and *NotI*, and the intervening region, which encodes amino acids 1477 to 1602, was replaced with DNA encoding the glycine linker and a HA tag to create BSKS-ArrowΔC.
The oligonucleotides used were:

Forward:

5'CGCGTGCTAGCGGAGGTGGCGGCGGAGGTGGAGGTGGCGGTTATCCTTATGACGTGC CTGACTATGCG

Reverse:

5'AATTCGCATACTGAGGCACGTCATAAGGATAACCGCCACCTCCACCTCCGCCGCCAC

CTCCGCTAGCA

BSKS-ArrowΔC was cut with *Asp718*, treated with the Klenow fragment of DNA polymerase I and then cut with XbaI to excise the ArrowΔC fragment.

pUAST was cut with *EcoRI*, treated with the Klenow fragment of DNA polymerase I and then cut with *XbaI* to create compatible ends for the ArrowΔC fragment. The two fragments were ligated and sequencing was used to verify its identity (Oswel Research).

Arrow-PPAP

To generate Arrow-PPAP, a DNA molecule was synthesized encoding the cytoplasmic tail of Arrow, modified so that the five PPP(S/T)P were mutated to PPPAP and a HA tag was added (synthesis by GenScript). BSKSArrow was digested with *NotI* and *SnaBI*, and the synthesized DNA inserted to create BSKSArrow-PPAP.

BSKS-Arrow-PPAP was cut with *Asp718*, treated with the Klenow fragment of DNA polymerase I and then cut with XbaI to excise the Arrow-PPAP fragment.

pUAST was cut with *EcoRI*, treated with the Klenow fragment of DNA polymerase I and then cut with *XbaI* to create compatible ends for the Arrow-PPAP fragment. The two fragments were ligated and sequencing was used to verify its identity (Oswel Research).

Synthesized DNA sequence:

AAGAATTCCTACGATCGCAATCATATCACAGGCGCCTCAAGCTCAACGACGAATGGTA
GCAGTATGGTGGCGTATCCAATCAATCCACCACCGGCGCCAGCAACCAGATCGCGTCG
CCCGTACAGGCACTACAAGATCATCAATCAGCCTCCACCGCCAGCACCCTGCTCAACG
GACATTTGTGATGAGTCTGACTCGAATTATACGAGCAAATCGAACAGCAATAACAGTA
ATGGGGGAGCCACAAAGCATTCTTCCAGTTCGGCGGCCGCCTGCTTGCAATATGGCTA
CGATAGTGAGCCGTATCCGCCGCCGCCGGCACCACGCTCCCATTACCACAGCGATGTG
CGCATTGTGCCGGAATCCTCCTGCCCGCCAGCGCCGTCATCGCGGAGCTCCACGTACT

TCTCGCCGCTCCCGCCTCCGCCAGCACCAGTACAGTCGCCAAGTCGGGGATTTTATCC
TTATGACGTGCCTGACTATGCGGCTACGTAA

Arrow ΔLysine

To generate ArrowΔLysine, a DNA molecule was synthesized encoding the cytoplasmic tail of Arrow, modified so that the bases that encoded lysines encoded alanines and a HA tag was added. Arrow BSKS was digested with *MluI* and *SnaBI*, and the synthesized DNA inserted. pUAST was cut with *EcoRI*, treated with the Klenow fragment of DNA polymerase I and then cut with *XbaI* to create compatible ends for the Arrow-ΔLysine fragment. The two fragments were ligated and sequencing was used to verify its identity (Oswel Research).

Synthesized DNA sequence:

ACGCGTATTGGAGCGAGTCGAACAGAGCCTGCGGATGACCAGGCCACTGATCCATTGT
CACCCTCGACGCTGAGCGCATCGCAAAGGGTTTCCGCAATCGCTTCGGTTGCGGATGC
AGTACGCATGTCTACGCTGAACTCGCGCAACAGCATGAATTCCTACGATCGCAATCAT
ATCACAGGCGCCTCAAGCTCAACGACGAATGGTAGCAGTATGGTGGCGTATCCAATCA
ATCCACCACCGTCGCCAGCAACCAGATCGCGTCGCCCGTACAGGCACTACGCGATCAT
CAATCAGCCTCCACCGCCAACACCCTGCTCAACGGACATTTGTGATGAGTCTGACTCG
AATTATACGAGCGCATCGAACAGCAATAACAGTAATGGGGGAGCCACAGCGCATTCTT
CCAGTTCGGCGGCCGCCTGCTTGCAATATGGCTACGATAGTGAGCCGTATCCGCCGCC
GCCGACACCACGCTCCCATTACCACAGCGATGTGCGCATTGTGCCGGAATCCTCCTGC
CCGCCATCGCCGTCATCGCGGAGCTCCACGTACTTCTCGCCGCTCCCGCCTCCGCCAT
CACCAGTACAGTCGCCGAGTCGGGGATTTTATCCTTATGACGTGCCTGACTATGCGGC
TACGTAA

CHAPTER 3 – WINGLESS ENDOCYTOSIS IN THE WING IMAGINAL DISC

3 CHAPTER 3 - WINGLESS ENDOCYTOSIS IN THE WING IMAGINAL DISC

3.1 Introduction

Prior to targeting to lysosomes, Wingless must be first captured at the cell surface and subsequently internalised. Binding of Wingless to its receptors has been investigated biochemically. Frizzled, Dfz2 and DFrizzled-3 all show binding activity, with Dfz2 exhibiting the highest affinity (Wu and Nusse, 2002). This study also investigated the binding of Wingless to Arrow, but no interaction was observed. However, a number immuno-precipitation experiments have reported binding between Wnts and LRP5/6 (Itasaki et al., 2003; Kato et al., 2002; Liu et al., 2003; Mao et al., 2001; Tamai et al., 2000; Tamai et al., 2004), suggesting that binding can occur between LRPs and Wnts.

It is likely that the Wingless receptors mediate the internalisation of Wingless. Internalisation of mammalian Frizzled4 requires β-Arrestin 2 and requires Dishevelled (Chen et al., 2003). Moreover, internalisation of Frizzled4 is stimulated by the presence of Wnt5a. However, as yet there is no evidence that Frizzled proteins can mediate endocytosis of Wnts. Arrow belongs to the LDL family of receptors and members of this family are implicated in binding and uptake of many factors including lipoprotein, proteases, and antibiotics (Herz and Bock, 2002; Nykjaer and Willnow, 2002). However, as yet no endocytic function has been attributed to Arrow and it lacks the conserved NPXY endocytic motif, present in members of the LDL family. The work in this chapter will investigate the role of Dfz2 and Arrow in the endocytosis of Wingless.

3.2 Dfz2 is endocytosed with Wingless

Overexpression of Dfz2 in the wing imaginal disc leads to stabilisation of Wingless (Cadigan et al., 1998) and the same effect is observed in the embryonic epidermis of *Drosophila*, although the effect is transient (Lecourtois et al., 2001). This observation has been used to suggest that Dfz2 could protect Wingless from degradation (Cadigan et al., 1998), possibly by sequestering or inhibiting an extracellular protease (Eldar et al., 2003).

It is possible that an extracellular protease could contribute to Wingless degradation. However, mutant clones of *deep-orange*, (the fly homolog of yeast Vps18 which is required for the trafficking of proteins to lysosomes (Sevrioukov et al., 1999)) in the wing disc accumulate Wingless protein (Laurence Dubois personal communication). This suggests that a substantial amount of Wingless is degraded in a lysosomal compartment.

Overexpression of Dfz2 could conceivably stabilise Wingless by blocking its endocytosis and trapping Wingless at the cell surface thus preventing subsequent routing to lysosomes. I investigated this possibility in imaginal discs.

First, I examined the distribution of Wingless in wild-type discs. In order to identify endocytic structures, discs were bathed in fluorescent dextran for 10 minutes, which is internalised by endocytosis, washed, incubated at room temperature for 20 minutes to allow endocytosis and then subsequently fixed and processed for immuno-fluorescence (Entchev et al., 2000). In wild-type discs, Wingless punctae are observed up to 7-8 cells from the Wingless producing cells and 66.01% of dextran labelled structures in this domain colocalise with Wingless (Table 3.1). Therefore, Wingless is present in endocytic structures.

To further investigate the composition of these structures, I looked at the relative subcellular distributions of Wingless, Dfz2 and dextran. The Gal4/UAS system (Brand

and Perrimon, 1993) was used to ectopically express a FLAG tagged full-length version of Dfz2 in the Dpp domain, which lies adjacent to A-P boundary of the disc, perpendicular to the Wingless expressing cells (Figure 3.1).

As previously shown, Wingless is stabilised by Dfz2 over a distance of 7-8 cells on either side of the Wingless source (Cadigan et al., 1998) (Figure 3.2a). Wingless is observed to accumulate extracellularily (Appendix 1) and also in punctate structures in cells overexpressing Dfz2 (arrows in Figure 3.2b). These structures often colocalise with Dfz2-FLAG and Dextran (arrows in Figure 3.2b-b'''). Quantitation of this observation showed that, 58.44% of the Dextran positive structures in the region where Wingless is stabilised colocalise with Wingless and Dfz2. Only a small number of Dextran positive structure that colocalise with Wingless do not contain Dfz2 (3.89%. Table 3.1) suggesting that Wingless is primarily endocytosed with Dfz2. It should be noted that overexpression of Dfz2 does not increase the rate of Wg endocytosis compared to the wild-type (Table 3.1) indicating that Dfz2 does not appear to stimulate further Wg endocytosis. In order to further investigate the distribution of Wingless in cells overexpressing Dfz2, I examined the apical-basal distributions of Wingless, Dfz2-FLAG and dextran (Figure 3.3). Overexpressed Dfz2 accumulates on the apical surface of imaginal disc cells and is also present basolaterally (Figure 3.3c') and Wingless stabilised by Dfz2 is primarily observed basolaterally (Figure 3.2') as previously reported (Strigini and Cohen, 2000). Punctate structures that contain Wingless, Dfz2 and Dextran are observed in the basolateral domain of the disc (Arrows Figures 3.3a'-d').

The colocalisation of Dfz2, Wg and dextran implicates Dfz2 in the endocytosis of Wingless; however, its exact role in Wg endocytosis is unclear. I investigated this by assessing whether various mutant forms of Dfz2 colocalise with Wingless in endosomes.

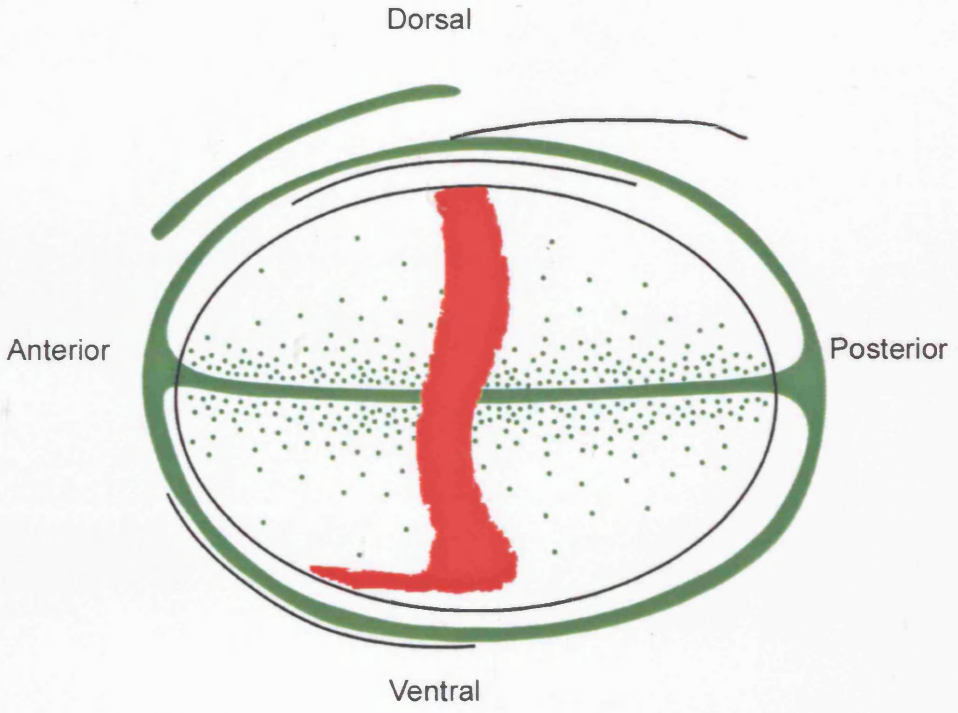


Figure 3.1 Schematic showing the expression patterns of Wingless and Dpp in the wing pouch

Wingless (green) is expressed at a stripe at the D-V boundary and in rings around the pouch. Dpp (red) is expressed at stripe adjacent to the A-P boundary, perpendicular to the Wingless expressing cells.

Figure 3.2 Dfz2 stabilises Wingless and colocalises with Wingless in endocytic structures

Imaginal discs of the genotype *dpp-gal4 UAS-Dfz2-FLAG* were isolated and bathed in 3kDa dextran-Texas Red for 10 minutes. After a 20 minute chase, they were fixed and stained. Low magnification of imaginal disc is shown in a-a''', high magnification of the boxed area in a-a''' is shown in b-b'''. Discs were labelled with anti-Wingless (a and b), Texas-Red-Dextran (a' and b') and anti-FLAG (a'' and b''). Images are single confocal sections in the basolateral domain where Wingless is stabilised (see also Figure 3.3)

(a-a''') Wingless is stabilised upon ectopic expression of Dfz2-FLAG.

(b-b''') Wingless is observed at the cell surface of cells expressing Dfz2-FLAG and in punctate structures that are labelled with dextran and Dfz2 (arrows in b-b''').

In all images, scale bars represent 10μm.

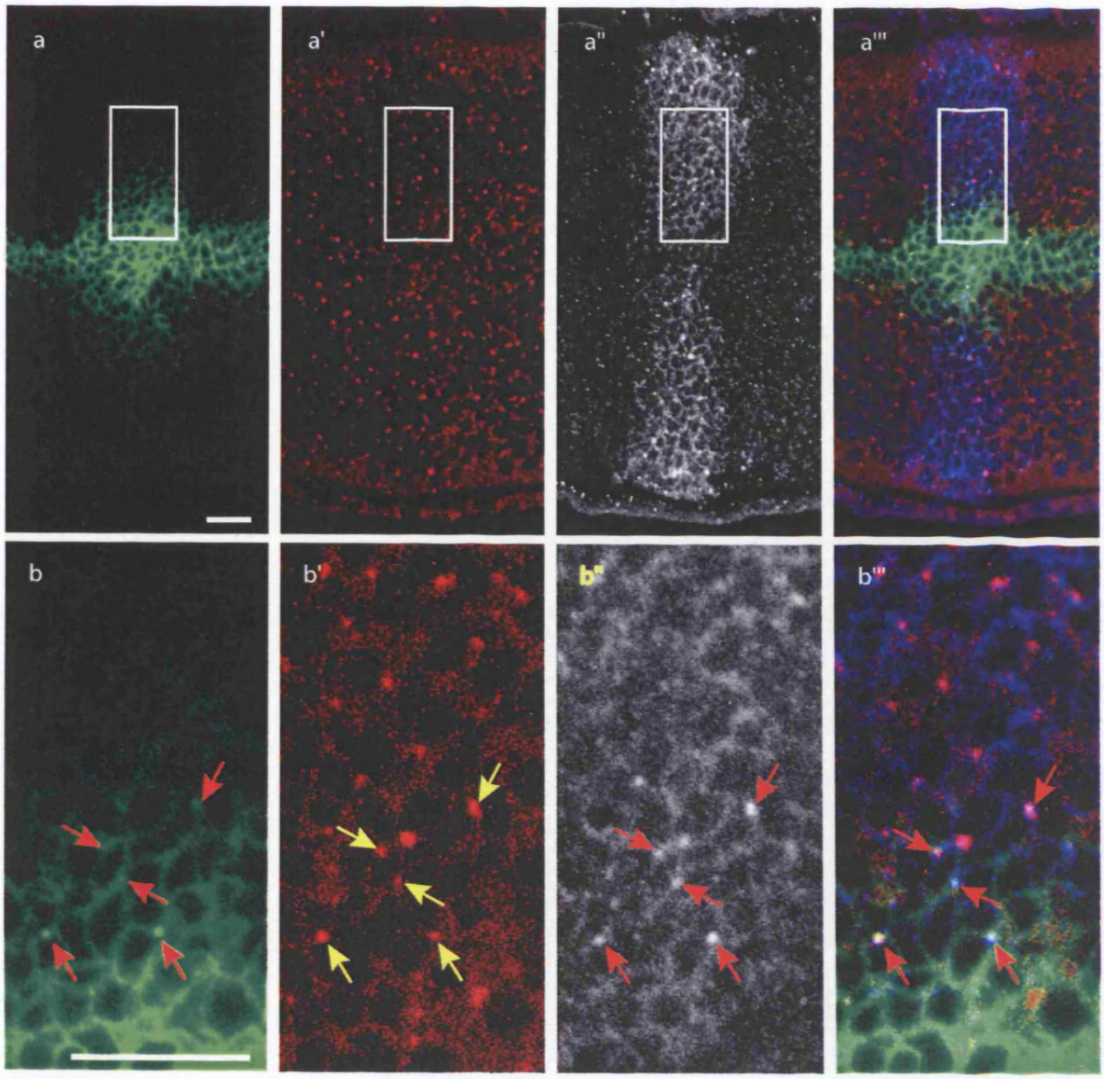
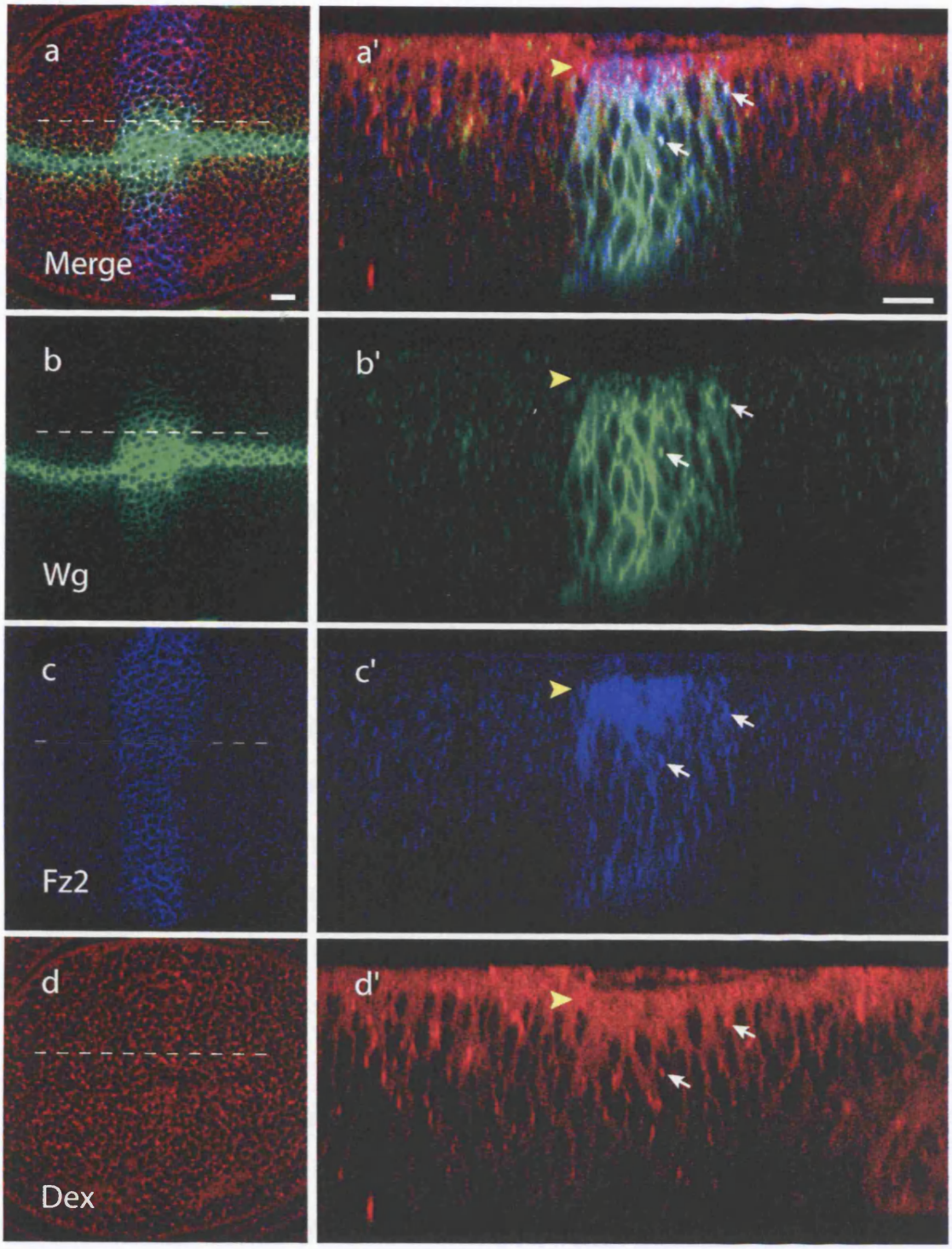

a
a'
a''
a'''
b
b'
b''
b'''

Figure 3.3 Z-section of wing disc expressing Dfz2-FLAG

Imaginal disc of the same genotype as Figure 3.2. Single confocal sections labelled with anti-Wingless (b and b'), anti-FLAG (c and c') and Texas-Red-Dextran (d and d'). Region of z-sections in a', b', c' and d' indicated by the dashed line in a, b, c and d.
a' Merged image of disc, apical domain indicated by yellow arrowhead.
b' Wingless is stabilised primarily in the basolateral domain. And is present at the cell surface and punctate structures that are labelled with dextran and Dfz2 (white arrows).
c' Dfz2-FLAG accumulates apically and is also present basolaterally.
d' Dextran positive structures are observed distributed throughout the disc.


a
Merge
a'
b
Wg
b'
c
Fz2
c'
d
Dex
d'

3.3 Truncating the cytoplasmic tail of Dfz2 reduces Wingless endocytosis

The cytoplasmic tail of Dfz2 is the likely location of possible endocytic signals. Therefore I assessed the distribution of Wingless and Dextran in wing discs expressing a form a DFz2 that lacks most of the intracellular tail (Dfz2AV) and is tagged by the FLAG-tag epitope.

Firstly, it was observed that when Dfz2AV is expressed, Wingless is stabilised over a much larger range than when full-length D-Frizzled 2 is expressed (Figure 3.4a). Secondly, while Wingless is still present at the cell surface, there is a marked reduction in the number of punctate Wingless structures observed (Figure 3.4a and 3.4b). Analysis of the Dextran and Dfz2AV distribution confirmed this. Of the dextran positive structures in the region where Wingless is stabilised, only 32% contain Wingless and Dfz2AV (red arrows in Figure 3.4b-b'''), compared to 58.44% when full-length Dfz2 is expressed (Table 3.1). Colocalisation of Wingless with dextran either in the presence or absence of Dfz2AV was reduced to 38.38% compared to 66.01% in the wild-type situation (Table 3.1) suggesting an overall reduction in Wingless endocytosis in the presence of Dfz2AV.

The subcellular distribution of Dfz2AV is similar to that of Dfz2 full-length. Dfz2AV accumulates on the apical surface of the imaginal disc cells and is also present at lower levels in the basolateral surface (Figure 3.5a' and c'). In cells overexpressing Dfz2AV Wingless is essentially absent from the apical domain and is present on the basolateral surface (Figure 3.5a' and b'). It is therefore likely that the reduction in Wingless endocytosis by Dfz2AV is due to mislocalisation of the mutated receptor.

These results suggest that Dfz2 could internalise Wingless and that the cytoplasmic tail of Dfz2 could harbour some of the signals that bring about the endocytosis of Dfz2. It should be noted that Wingless endocytosis is not completely abolished upon Dfz2AV

expression (yellow arrows in Figure 3.4b-b'''). Therefore, there could be other signals in Dfz2 that are involved in endocytosis. Alternatively, other molecules could mediate the internalisation of Dfz2 and Wingless.

Figure 3.4 Truncating the cytoplasmic tail of Dfz2 reduces Wingless endocytosis

Imaginal discs of the genotype *dpp-gal4 UAS-Dfz2AV-FLAG* were isolated and bathed in 3kDa dextran-Texas Red as previously described. Low magnification of imaginal disc is shown in a-a'', high magnification of the boxed area is shown in b'-b''. Discs were labelled with anti-Wingless (a and b), Texas-Red-Dextran (a' and b') and anti-FLAG (a''and b''). Images are single confocal sections in the basolateral domain where Wingless is stabilised (see also Figure 3.5)

(a-a'') Wingless is stabilised upon ectopic expression of Dfz2AV-FLAG.

(b-b''')Wingless is observed at the cell surface of cells expressing Dfz2AV-FLAG dextran positive structures do not colocalise with Wingless and Dfz2AV (red arrows in b-b''), except in very few cases (yellow arrows in b-b'').

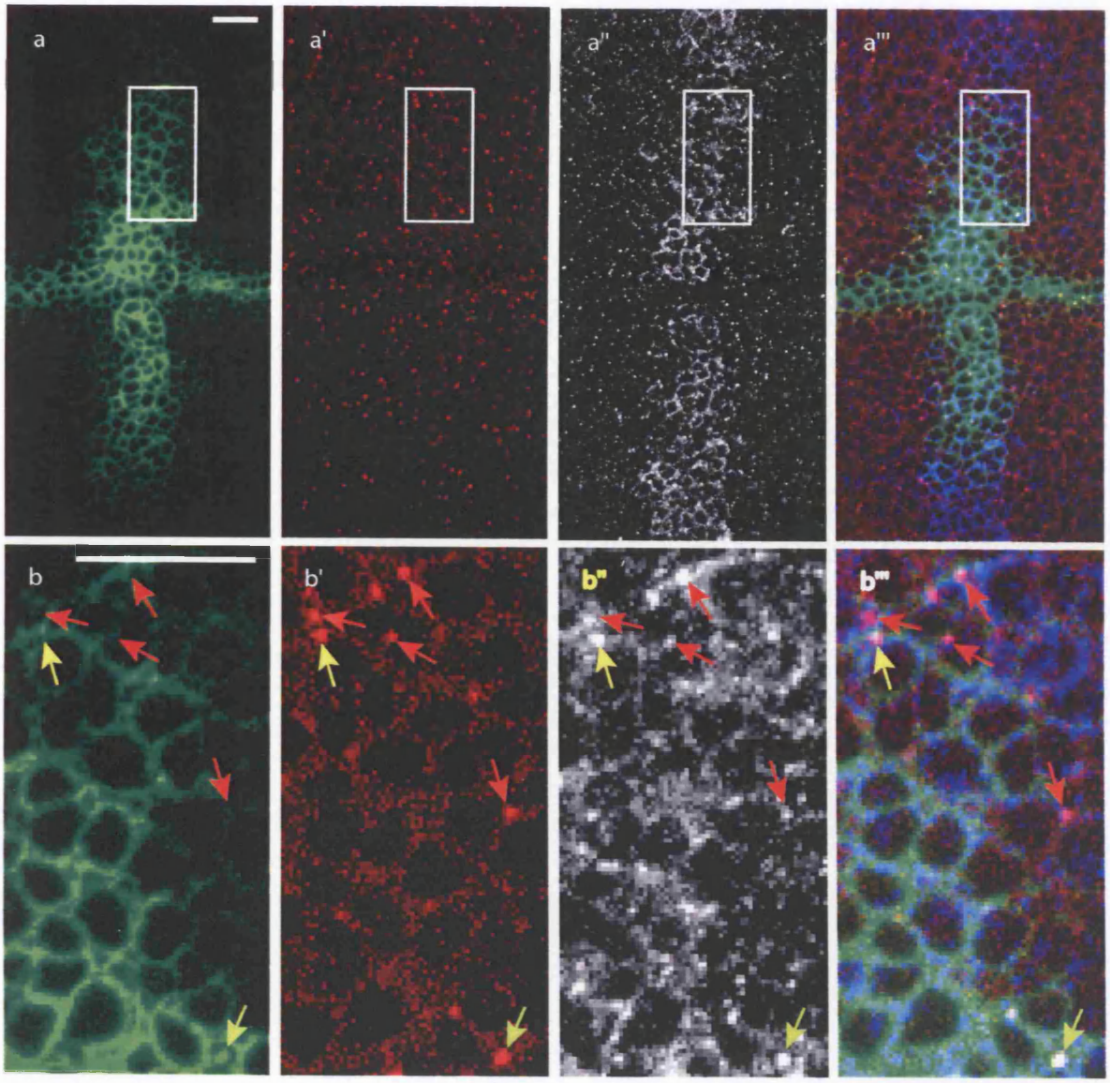

a
a'
a''
a'''
b
b'
b''
b'''

Figure 3.5 Z-section of wing disc expressing Dfz2AV

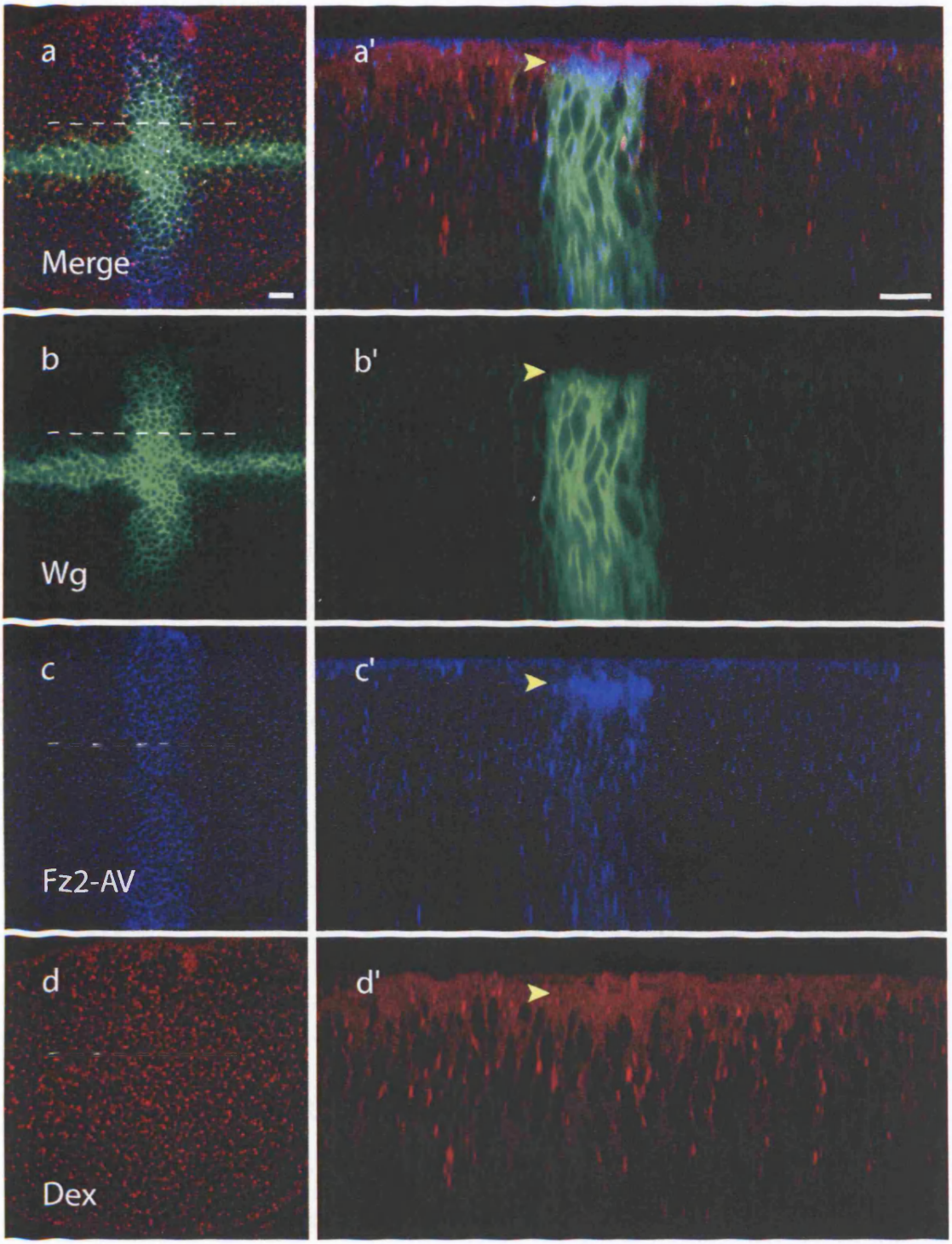
Imaginal disc of the same genotype as Figure 3.4. Single confocal sections of disc labelled with anti-Wingless (b and b'), anti-FLAG (c and c') and Texas-Red-Dextran (d and d'). Domain of z-sections in (a'), (b'), (c') and (d') indicated by the dashed line in a, b, c and d.

(a') Merged image of disc, apical domain indicated by yellow arrowhead.

(b') Wingless is stabilised primarily in the basolateral domain.

(c') Dfz2AV accumulates apically and is also observed basolaterally.

(d') Dextran positive structures are observed distributed throughout the disc.


a
Merge
a'
b
Wg
b'
c
Fz2-AV
c'
d
Dex
d'

3.4 Deletion of the Dfz2 transmembrane domains leads to a further reduction in Wingless endocytosis

To further investigate the domains of Dfz2 that regulate internalisation of Dfz2 and Wingless, I tested the ability of Dfz2-GPI to internalise Wingless. Dfz2-GPI is a form of Dfz2 that contains the ligand binding, N-terminal extracellular cysteine-rich domain of Dfz2 but lacks the transmembrane domains and the cytoplasmic tail; it is linked to the membrane by a GPI anchor (Cadigan et al., 1998).

As with the previous experiments, Dfz2-GPI was expressed in the Dpp domain and the discs were labelled with fluorescent dextran. Expression of this form of Dfz2 leads to stabilisation of Wingless (Figure 3.6a) and as with the Dfz2AV deletion, expression of Dfz2-GPI reduces the rate of Wingless endocytosis. Of the dextran positive structures, only 8% colocalise with Wingless and Dfz2-GPI (red arrows in Figure 3.6b-b''') (Table 3.1). However, occasionally Wingless is observed to colocalise with dextran and Dfz2-GPI (yellow arrows in Figure 3.6b-b'''). As deletion of all of the residues after the extracellular domain of Dfz2 is likely to remove all possible endocytic signals, it is likely that in these cases Wingless and Dfz2-GPI internalisation is either being mediated by an alternative molecule or possibly by intrinsic endocytosis of the GPI anchor. Wingless colocalisation with dextran is reduced compared to colocalisation in the presence of full-length Dfz2, but Wingless endocytosis is not completely abolished and Wingless and dextran are observed to that in the absence of Dfz2-GPI (Table 3.1). It is possible that in these cases, Wingless is being endocytosed with endogenous Dfz2 or by other molecules. Dfz2-GPI is observed to accumulate on the apical surface and is also present in the basolateral surface, it is also present at high levels on the basal surface (Figure 3.7a' and c'). As with Dfz2 full-length and Dfz2AV, stabilised Wingless is seen at the basolateral surface.

Figure 3.6 Dfz2-GPI does not internalise Wingless

Imaginal discs of the genotype *dpp-gal4/UAS-Dfz2GPI* were isolated and bathed in 3kDa dextran-Texas Red as previously described. Low magnification of imaginal disc is shown in a-a'', high magnification of the boxed area in a-a'' is shown in b'-b''. Discs were labelled with anti-Wingless (a and b), Texas-Red-Dextran (a' and b') and anti-c-Myc (a'' and b''). Images are single confocal sections in the basolateral domain where Wingless is stabilised (also see Figure 3.7)

(a-a'') Wingless is stabilised upon ectopic expression of Dfz2GPI. (b-b''')Wingless is observed at the cell surface of cells expressing Dfz2-GPI, but the dextran positive structures do not colocalise with Wingless and Dfz2-GPI (red arrows in b-b''') except in very few cases (yellow arrow in b-b''').

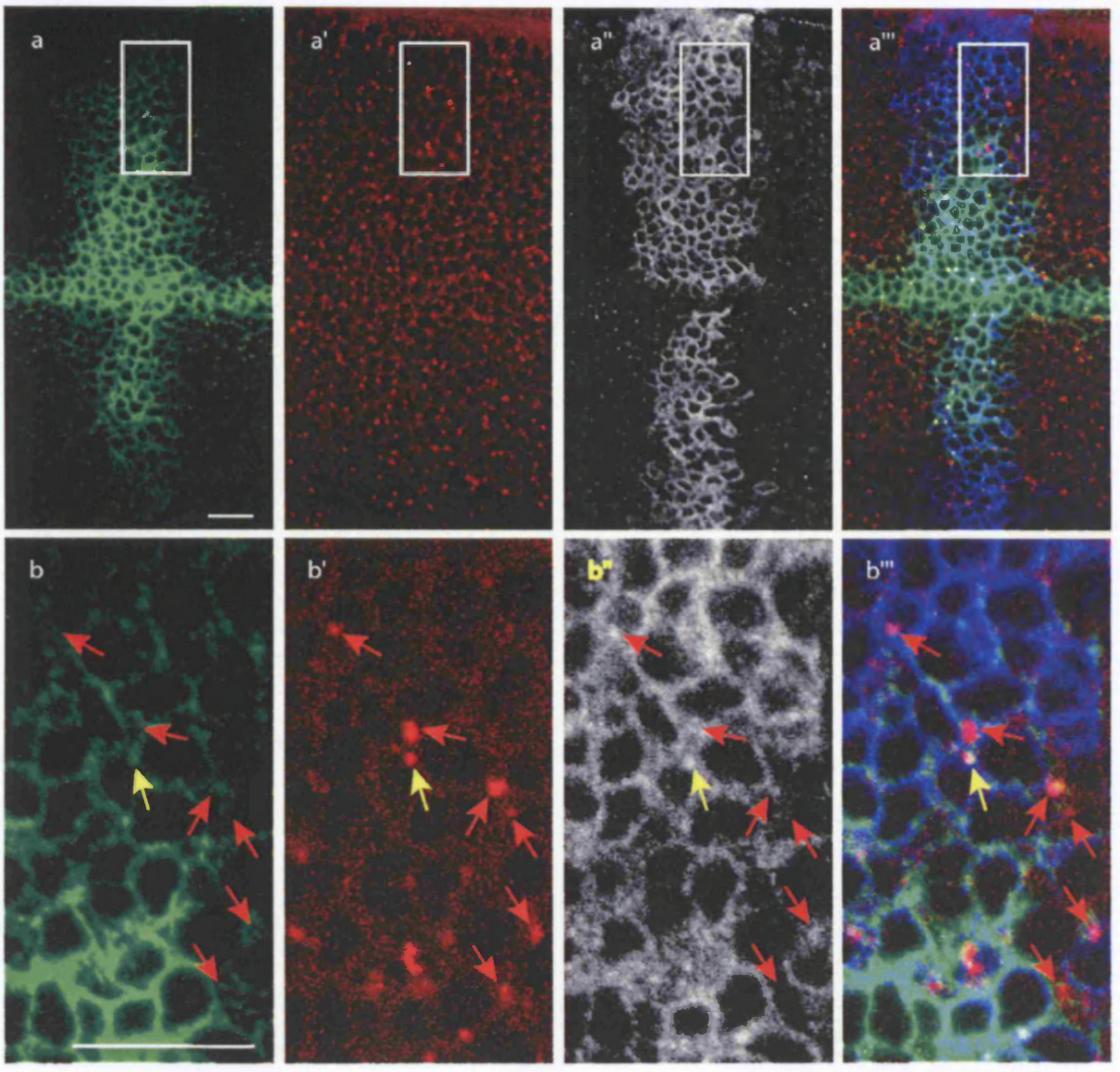

a
a'
a''
a'''
b
b'
b''
b'''

Figure 3.7 Z-section of wing disc expressing Dfz2-GPI

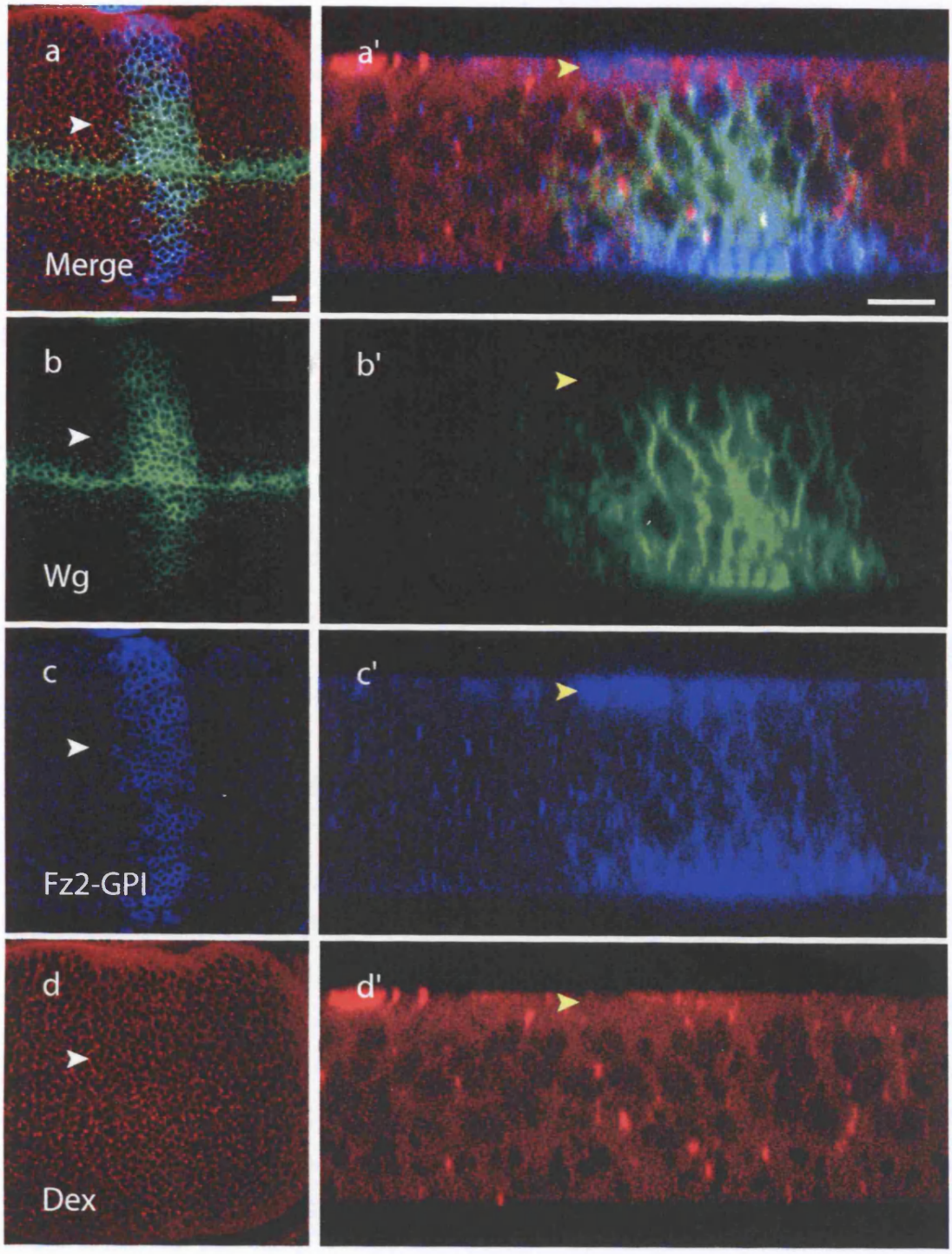
Imaginal disc of the same genotype as Figure 3.6. Single confocal sections of a disc labelled with anti-Wingless (b and b'), anti-FLAG (c and c') and Texas-Red-Dextran (d and d'). Domain of z-sections in a', b', c' an d' indicated by the dashed line in a, b, c and d.

(a') Merged image of disc, apical domain indicated by yellow arrowhead.

(b') Wingless is stabilised primarily in the basolateral domain

(c') Dfz2GPI accumulates on the apical surface and is also observed basolaterally and at particularly high levels in the most basal domain.

(d') Dextran positive structures are observed distributed throughout the disc.


a
Merge
a'
b
Wg
b'
c
Fz2-GPI
c'
d
Dex
d'

Together, these results indicate that Dfz2 harbours an endocytic signal and, by virtue of its ability to capture Wingless, it contributes to the targeting of Wingless into an endocytic compartment. Truncating the cytoplasmic and transmembrane domains of Dfz2 reduces Wingless endocytosis (Table 3.1).

	Dextran + Wg	Dextran + Wg + Fz2	Total
WT	66.01%	N/A	66.01%
Dfz2FL	3.89%	58.44%	62.34%
Dfz2AV	6.06%	32.32%	38.38%
Dfz2-GPI	14.71%	7.84%	22.55%

Table 3.1 Comparison of Wingless endocytosis by different forms of Dfz2

Percentages represent the colocalisation of Wingless and Dfz2 in dextran positive structures in cells located 6-7 cells from the source of Wingless. In wild-type tissue (WT), and when different forms of Dfz2 are expressed (Dfz2FL, Dfz2AV, Dfz2-GPI). The first column (Dextran +Wg) represents colocalisation of Dextran and Wingless alone. The second column (Dextran + Wingless + Fz2) represents dextran, Wingless and Dfz2 colocalisation. The third column (Total) The total colocalisation between Wingless and dextran either in the presence or absence of Dfz2.
Note the reduction of Wingless colocalisation with dextran and Dfz2AV and Dfz2GPI compared to full-length and the reduction in overall Wingless endocytosis in the presence of Dfz2AV and Dfz2-GPI.

3.5 Wingless transcription is unaffected in cells overexpressing Dfz2

Excess Wingless protein observed in cells that overexpress Dfz2 could be due to three different reasons. Firstly, as suggested in the previous experiments, it could be stabilised by being bound to Dfz2. Secondly, Wingless transcription could be activated

in the cells that express Dfz2, leading to increased protein levels. Thirdly, cells present in the normal Wingless expressing domain could move away from the D-V boundary, maintaining high levels of Wingless.

The cells that overexpress Dfz2 are likely to have higher than normal levels of Wingless signalling and, as Wingless signalling represses transcription of Wingless outside its normal domain of expression (Rulifson et al., 1996), it is unlikely that these cells ectopically express Wingless. I confirmed this expectation experimentally.

To do this, I crossed flies expressing Dfz2 in the Dpp domain to a reporter strain, WgLacZ. In these flies, when the Wingless promoter is active, β-Galactosidase protein is produced and reports on the activity of the Wingless promoter (Kassis et al., 1992). As can been seen in Figure 3.8, β-Galactosidase expression is not activated in the cells that stabilise Wingless. β-Galactosidase is considered to be relatively stable; therefore, this result also suggests that the third possibility mentioned above is unlikely. If the cells had moved out of the Wingless expression domain they would be recognised by the presence of β-Galactosidase, which is known to be perdure after transcription has stopped. As no β-Galactosidase is present in the cells that stabilise Wingless, it can be concluded that Wingless accumulates in the cells that overexpress Dfz2 as a result of stabilisation.

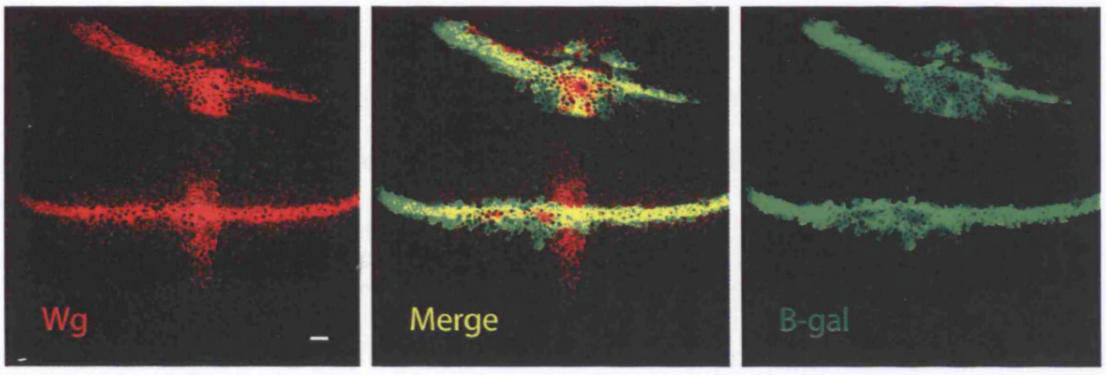


Figure 3.8 Cells ectopically expressing Dfz2 do not ectopically express Wingless

Imaginal disc of the genotype *wg-LacZ, UAS-Dfz2-FLAG, dpp-gal4*. Disc labelled with anti-Wingless (red), and anti β-Galactosidase (green) shows that cells expressing Dfz2-FLAG do not contain β-Galactosidase and therefore are not transcribing Wingless. Images are single confocal sections in the basolateral domain of the disc in a region where Wingless is stabilised by Dfz2.

3.6 Reducing Wingless Internalisation by Dfz2 reduces Wingless degradation

I previously described the three steps that are required in order for receptor-mediated degradation of an extracellular ligand. The results above demonstrate a role for Dfz2 in both the capture and endocytosis of Wingless. If Wingless is degraded after capture and internalisation by Dfz2, then it would be predicted that preventing Dfz2 endocytosis would consequently block Wingless degradation. Comparison of the range of stabilised Wingless between full-length Dfz2, Fz2AV and Dfz2-GPI suggests that this is indeed the case. On expression of full-length Dfz2, Wingless is stabilised 7-8 cells away from the Wingless source, whereas expression of Dfz2AV stabilises Wingless 12-13 cells away from the source (Figure 3.9).

The dextran labelling indicated that Dfz2-GPI is the least efficient form of Frizzled-2 with regards to Wingless endocytosis. This suggests a correlation between a reduction in endocytosis and stabilisation (Figure 3.9).

Expression levels of proteins driven by the Gal4-UAS system can vary depending on the location of the UAS-X insertion (Phelps and Brand, 1998). Therefore, it could be the increased stabilisation observed by the mutant forms of Dfz2 is due to higher expression levels of the corresponding transgenes. In order to assess this possibility I measured the level of receptor expressed in various situations: one copy of the UAS-Fz2-FLAG transgene, two copies of the UAS-Fz2-FLAG transgene and one copy of the Dfz2-GPI transgene. I used an anti-Dfz2 antibody targeted to the extracellular domain of Dfz2 that recognises all of the Dfz2 variants that I have used in these experiments (however, it is not sufficiently sensitive to recognise endogenous Dfz2). In all three experiments, the extent of Wingless stabilisation was assessed.

When two copies of the UAS-Dfz2 (full-length) transgene are present there is a small increase in the range of Wingless (Figure 3.10a and b). However, the extent of

stabilisation is still much less than by Dfz2-GPI (Figure 3.7 a-c). Comparison of the levels of Dfz2 expressed in each of these experiments indicates that when two copies of Dfz-2 are present, the amount of Fz2 expressed is increased when compared to the expression from one copy (compare Figure 3.10d and e). As the range of Wingless is not significantly increased with two copies of Dfz2 (Figure 3.10a and b). I suggest that increasing the amount of Dfz2 does not significantly increase the range of stabilised Wingless. The anti-DFz2 staining of discs expressing Dfz2-GPI indicates that it is expressed at a much lower level than full-length Dfz2 (compare Figure 3.10d to f), demonstrating hat increased stabilisation by Df2-GPI is not due to an increased level of receptor.

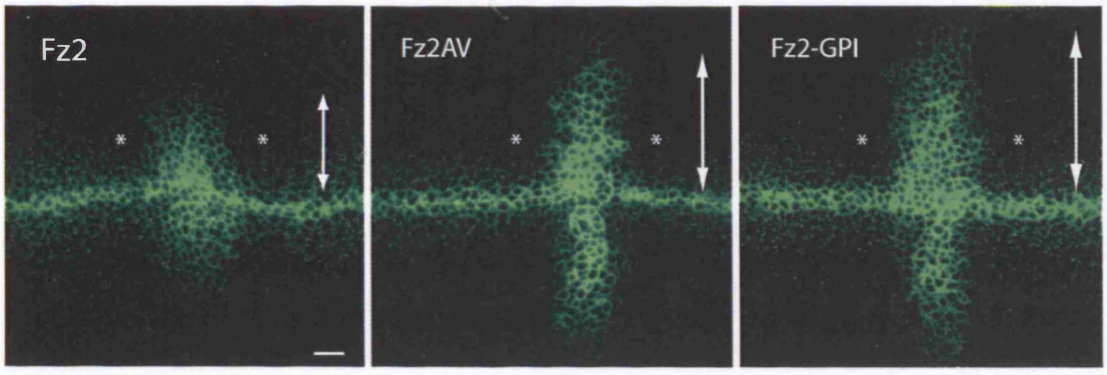


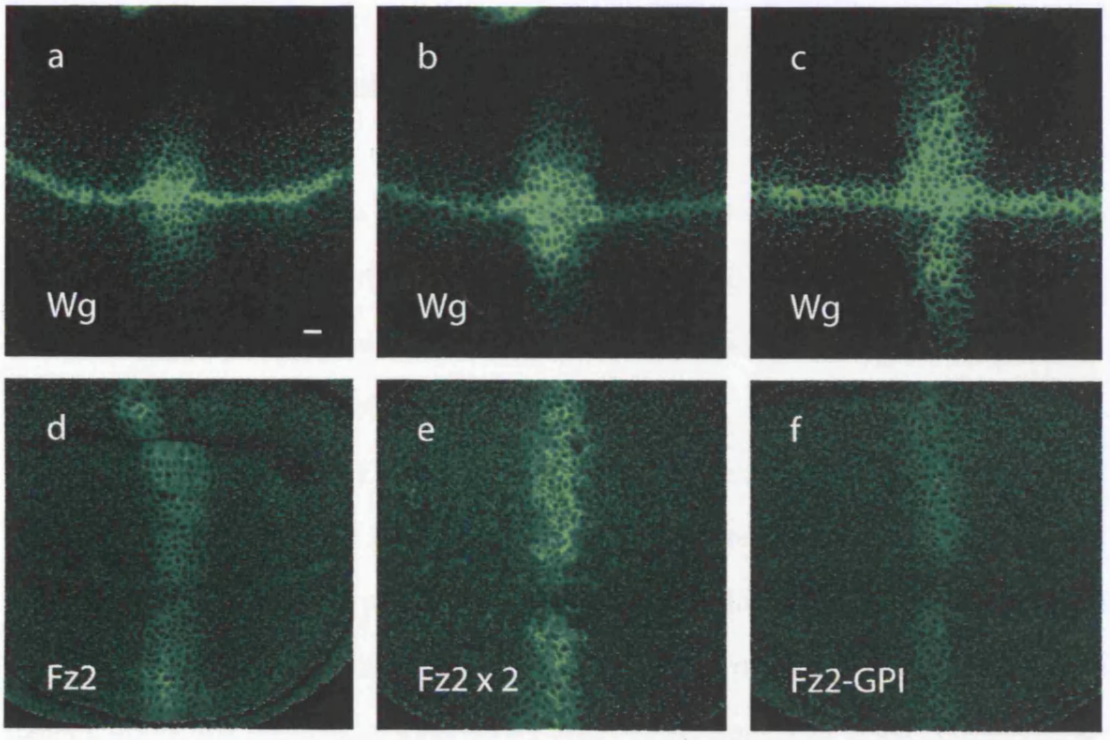
Figure 3.9 Truncating Dfz2 increases the range over which Wingless is stabilised

Imaginal discs labelled with anti-Wingless. Genotypes are *dpp-gal4 UAS-Dfz2-FLAG* (left panel), *dpp-gal4 UAS-Dfz2AV-FLAG* (centre panel) and *dpp-gal4 UAS-Dfz2-GPI* (right panel). Images are single confocal sections in the basolateral domain.
Wingless is stabilised over 7-8 cells by full-length Dfz2 (left panel), over 12-13 cells by Dfz2AV (centre panel) and over 17-18 cells by Dfz2-GPI (right panel). Also note the reduction of Wingless staining in the areas just adjacent to the region of Dfz2 expression (asterisks in all panels).

Figure 3.10 Increased Wingless stabilisation by Dfz2-GPI is not due to the dosage

Discs shown in (a) and (d) are of the genotype *dpp-gal4 UAS-Dfz2-FLAG*. Discs shown in b and e are of the genotype *dpp-gal4 UAS-Dfz2-FLAG/UAS-Dfz2-FLAG* and discs shown in c and e are of the genotype *dpp-gal4/UAS-fz2GPI*. Imaginal discs labelled with anti-Wingless (a-c) or anti-Dfz2 (d-f). Anti-Dfz2 is targeted to the first extracellular domain of Dfz2 and recognises all forms of Dfz2. All images are of single confocal sections in the basolateral domain.

When two copies of UAS-Fz2 are present, the range of Wingless is not significantly increased (compare a and b), even though there is an increase in the amount of Dfz2 present (compare d and e). Wingless is stabilised over a wider range by Dfz2-GPI than by full-length Dfz2 (compare a and c) even though the level of Dfz2-GPI expression is lower than that of full-length Dfz2 (compare d and e).


a
Wg
b
Wg
c
Wg
d
Fz2
e
Fz2 x 2
f
Fz2-GPI

3.7 Wing phenotypes of Dfz2 constructs

Flies that ectopically express FLAG tagged, full-length Dfz2 in the Dpp domain of the wing disc are viable and fertile. The wings show ectopic bristles close to the margin (compare Figure 3.11a and a' with 3.11b and b') an indication of increased Wingless signalling see also(Cadigan et al., 1998).

Interestingly, expression of Dfz2AV, which lacks almost all of the cytoplasmic tail of Dfz2, is still able to activate signalling. Ectopic bristles are observed in the adult wings of flies that express this form of Dfz2 in the Dpp domain (Figure 3.11c and c'). The short cytoplasmic tail of Dfz2AV contains the KTxxxY motif, which is implicated in signal transduction by Dfz2 proteins (Cong et al., 2004; Umbhauer et al., 2000).

The wings of flies that express Dfz2-GPI in the Dpp domain in almost all cases resemble wild-type flies. Very rarely (<5%) adult flies exhibit notches in the wing (Figure 3.11d), a phenotype associated with the loss of Wingless signalling. In these cases it appears that Dfz2-GPI is acting as a dominant-negative, as has been previously reported (Cadigan et al., 1998). As Dfz2-GPI lacks all the transmembrane and intracellular residues of Dfz2, it is likely that, while it can still bind Wingless, it cannot associate with the downstream components of the signalling pathway.

Figure 3.11 Wing phenotypes of Dfz2 expressing flies

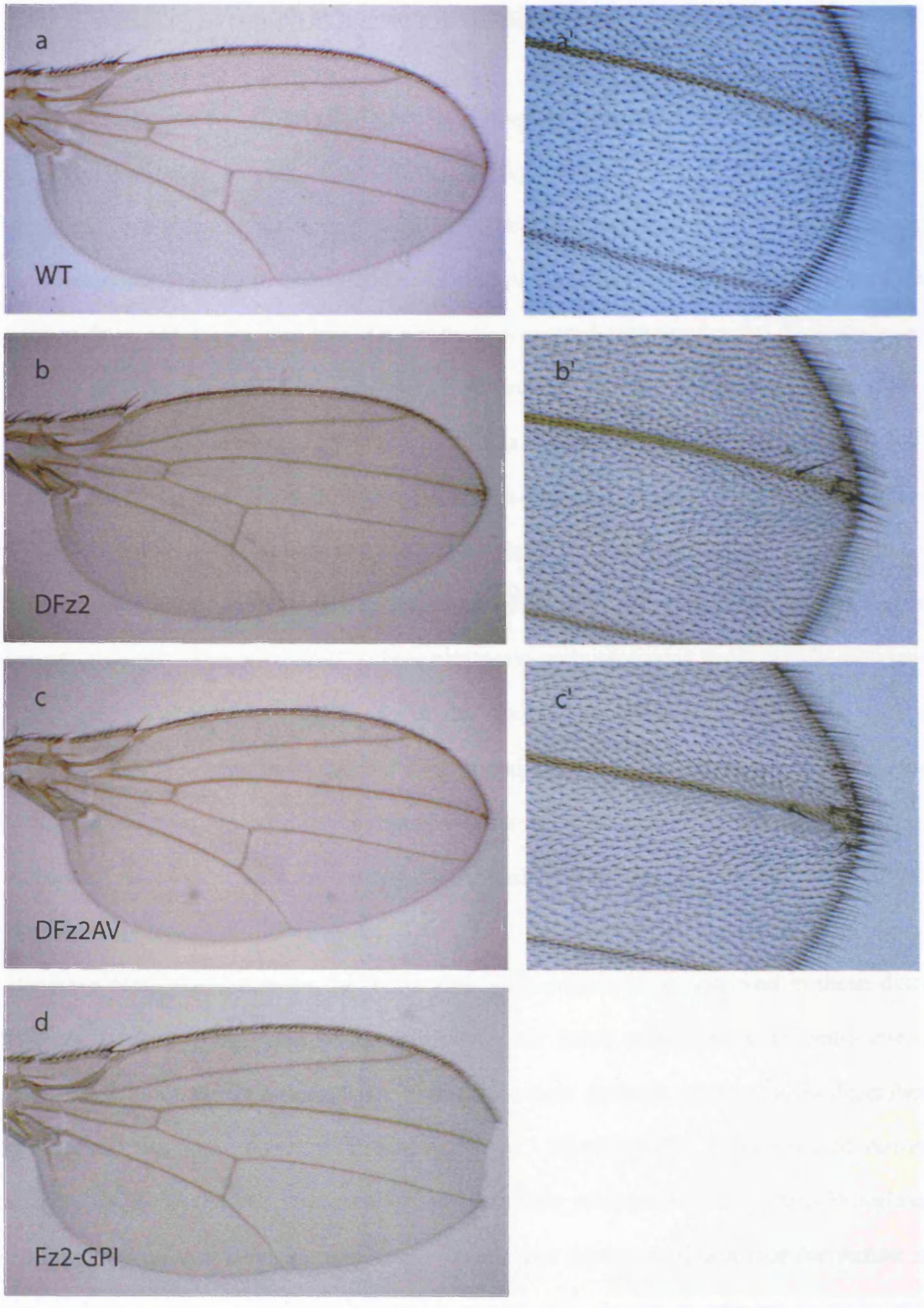
Genotypes: wild-type (a-a'), *dpp-gal4 UAS-Dfz2-FLAG* (b-b'), *dpp-gal4 UAS-Dfz2AV-FLAG* (c-c') and *dpp-gal4 UAS-Dfz2-GPI* (d).

(a) Wild-type wing (shown in high magnification in a')

(b) Ectopic expression of Dfz2-FLAG leads to ectopic bristle formation (shown in high magnification in b'), a phenotype associated with increased Wingless signalling.

(c) Ectopic expression of Dfz2AV also leads to ectopic bristle formation, (shown in high magnification in c'). This confirms that truncating the c-terminus tail of Fz2 does not prevent Wingless signalling.

(d) Ectopic expression of Dfz2-GPI can cause notches in the wing, a phenotype associated with a loss of Wingless signalling. This phenotype is only exhibited in ~5% of flies of this genotype.


a
WT
a'
b
DFz2
b'
c
DFz2AV
c'
d
Fz2-GPI

3.8 Effect of Arrow on Wingless distribution

In order to assess the effect of Arrow on Wingless distribution, I generated a DNA construct encoding a form of arrow that is tagged with the HA epitope at the C-terminus. This was subcloned into the pUAST vector, downstream of the UAS sites and introduced into flies by microinjection of *Drosophila* embryos. The *dpp-gal4* driver was used to drive expression and anti-HA antibody was used to recognise the overexpressed protein (Figure 3.12a-a''). Expression of Arrow in the Dpp domain does not affect Wingless distribution significantly (Figure 3.12a); this could possibly be due to its low affinity for Wingless (Wu and Nusse, 2002). Z sections of discs ectopically expressing Arrow show that Arrow is present in the basolateral domain of the disc (3.12b-b')

The Arrow-HA construct is clearly active, as overexpression causes ectopic bristles in the adult wings (Figure 3.12c-c''). This was previously observed for other UAS-Arrow transgenes (Wehrli et al., 2000). As Arrow has a low affinity for Wingless, it was possible that expression in the narrow Dpp domain would not allow subtle effects on the Wingless distribution to be detected. In order to assess whether this is the case, I expressed Arrow-HA with *apterous-gal4* (*ap-gal4*) driver that drives expression in the dorsal compartment of the wing disc.

Punctate Wingless structures that colocalise with Arrow were observed in these discs (Figure 3.13a-a''). In order to establish whether these structures were endocytic, I labelled discs of the same genotype with fluorescent dextran, as previously described (Figure 3.13b). The arrows in Figure 3.13b-b''' illustrate that Wingless and Arrow colocalise in endocytic structures. Of the punctate structures that contain Wingless, 52.5% contain both Dextran and Arrow. While not demonstrating a role for Arrow in the endocytosis of Wingless this result shows that Arrow is present in endocytic structures with Wingless.

Figure 3.12 Phenotypes associated with ectopic expression of Arrow

(a-a') Wing discs of the genotype *dpp-gal4, UAS-Arrow-HA* labelled with anti-Wingless (a) and anti-HA (a'). Wingless distribution is unaffected by expression of Arrow. Images are single confocal sections in the basolateral domain of the disc.
(b-b') Wing disc of same genotype as in (a) shown in optical cross-section (b'). the dashed line in (b) indicates the region of the cross section. Te apical domain is indicated with the arrowhead in (b') Arrow-HA is present basolaterally.
(c) Adult wing of the genotype *dpp-gal4, UAS-Arrow-HA*. Ectopic expression of Arrow-HA leads to ectopic bristle formation, (shown in high magnification in c'), indicating that ectopic expression of Arrow leads to increased activation of the Wingless pathway.

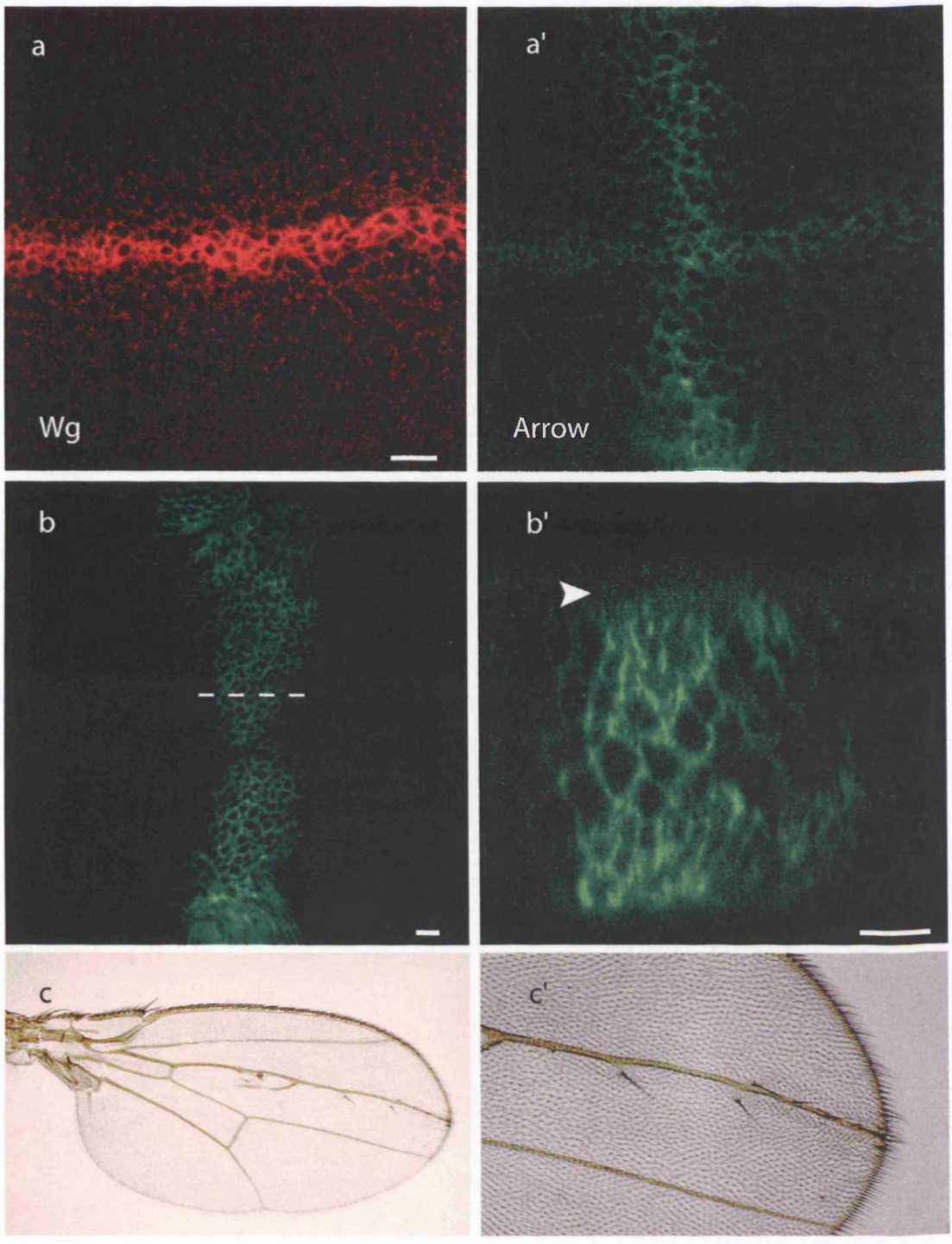
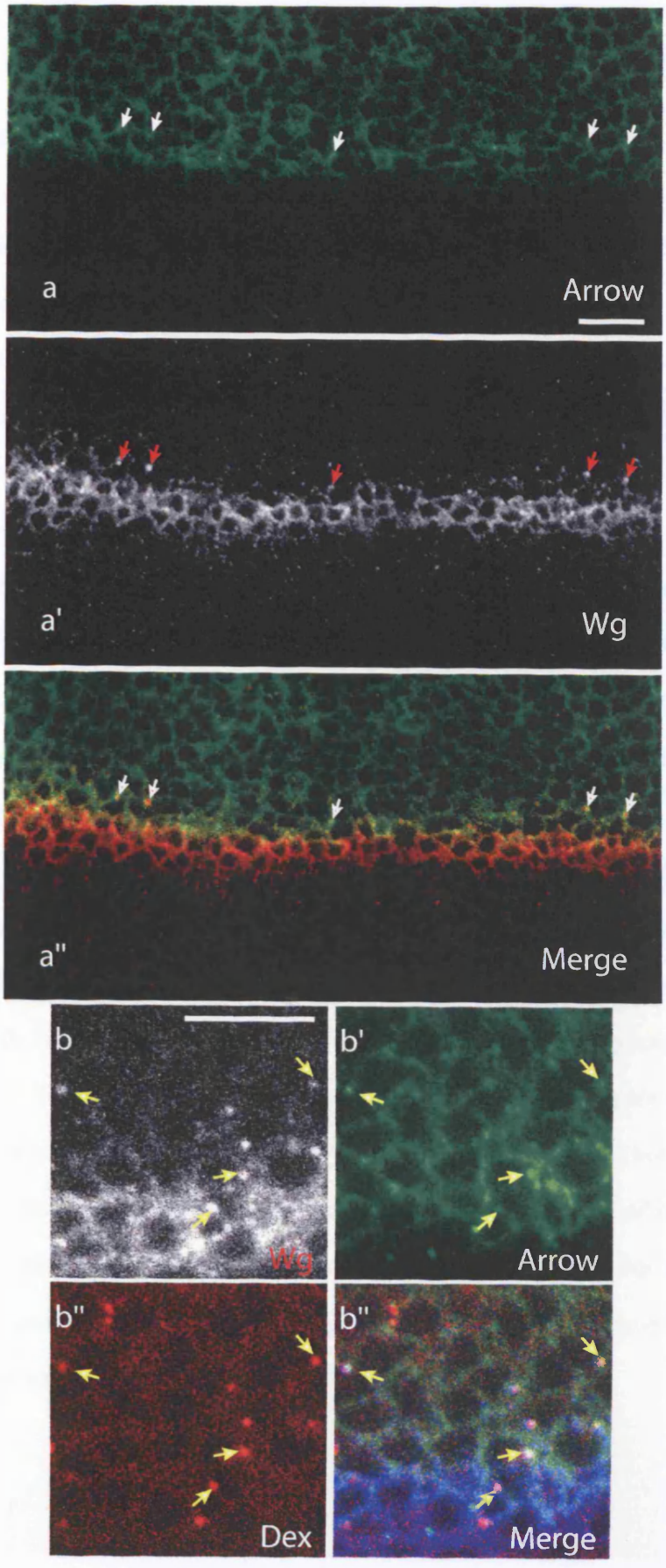

a
Wg
a'
Arrow
b
b'
c
c'

Figure 3.13 Arrow colocalises with Wingless in endocytic structures

Wing discs of the genotype *ap-gal4 UAS-Arrow-HA*. Single basolateral confocal sections.

(a-a'') Punctate Wingless structures observed in the domain of ectopic Arrow expression colocalise with Arrow-HA (Arrows).

(b-b''') Endocytic structures labelled with dextran (b'') contain Wingless (b) Arrow (b') (arrows). Merge of the three channels is shown in (b''')


a
Arrow
a'
Wg
a''
Merge
b
Wg
b'
Arrow
b''
Dex
b''
Merge

3.9 Summary

- Ectopic expression of Dfz2 leads to basolateral Wingless accumulation.
- Wingless colocalises with Dfz2 in endocytic structures.
- Truncating Dfz2 at the c-terminus reduces Wingless endocytosis and increases the range of stabilised Wingless.
- Arrow also colocalises with Wingless in endocytic structures.

Together, these results suggest that Dfz2 plays an important role in the endocytosis of Wingless. Wingless is seen to colocalise with Dfz2 in endocytic structures and truncating the c-terminal tail of Dfz2 reduces Wingless endocytosis. From this, it is clear that overexpressed DFz2 does not stabilise Wingless by blocking its endocytosis, as has been previously suggested (Cadigan et al., 1998).

These results also suggest that that capture and endocytosis of Wingless by Dfz2 are steps in the pathway used to degrade Wingless, as blocking Wingless endocytosis by Dfz2 increases the amount of Wingless protein stabilised. Even though Arrow does colocalise with Wingless in endocytic structures, it's role in Wingless endocytosis remains unclear. This question will be addressed further in the subsequent chapters.

It is clear that, while it plays a role in endocytosis of Wingless, Dfz2 is not sufficient for Wingless degradation, as overexpression stabilises Wingless. One possible explanation for this is that after endocytosis by Dfz2, another factor, required for trafficking to lysosomes, becomes limiting in the presence of excess Dfz2. This possibility will be addressed in the next chapter.

CHAPTER 4 – WINGLESS DEGRADATION IN THE WING DISC

4 CHAPTER 4 - WINGLESS DEGRADATION IN THE WING DISC

4.1 Introduction

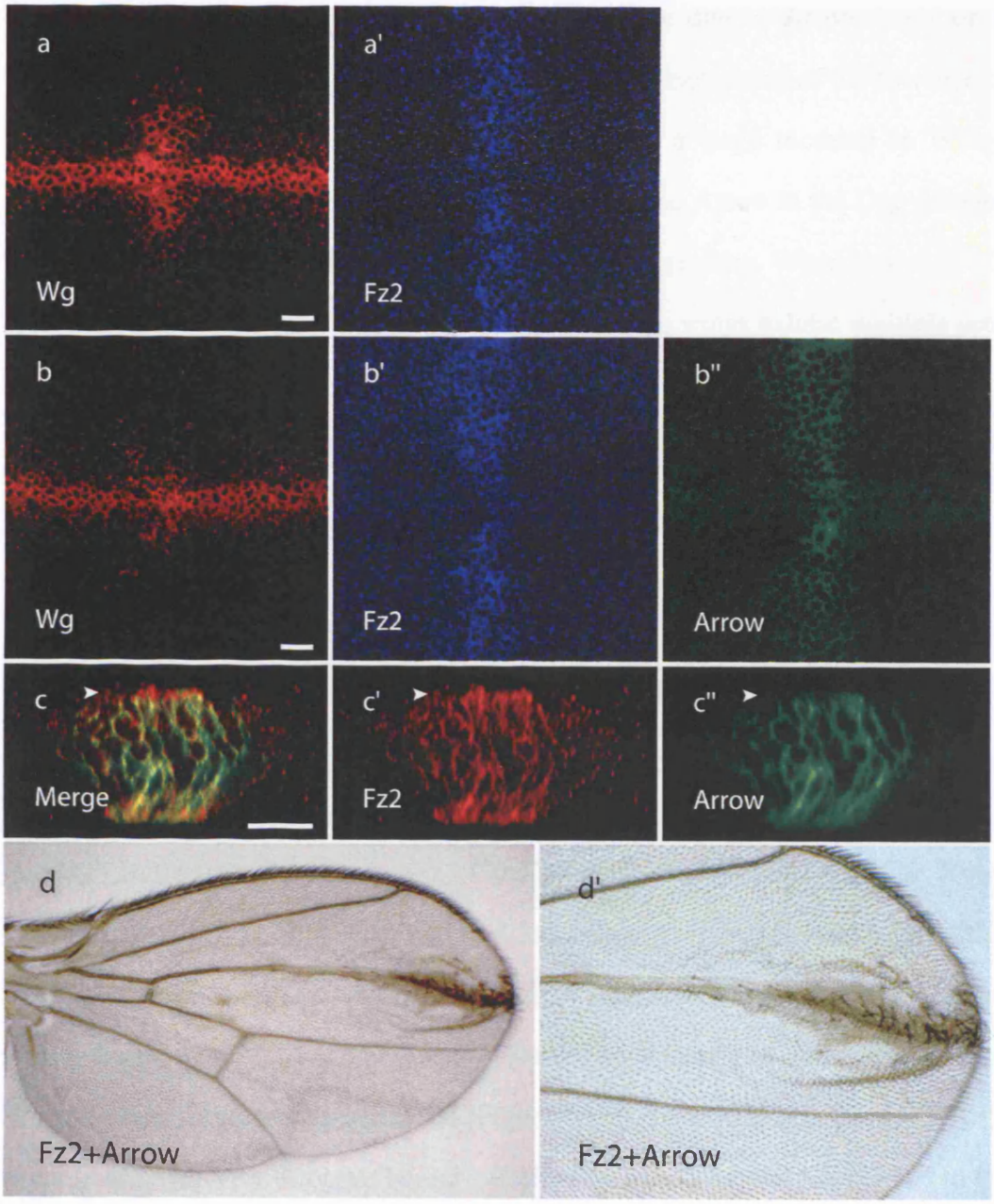
The results outlined in the previous chapter suggest that Dfz2 plays a key role in the endocytosis of Wingless and that endocytosis of Wingless by Dfz2 results in Wingless degradation. However, it is clear that Dfz2 is not sufficient for Wingless degradation, as Wingless is stabilised upon ectopic expression of Dfz2. A possible explanation for this is that another factor, required for trafficking to lysosomes after endocytosis, becomes limiting in the presence of excess Dfz2. One factor that could function with Dfz2 is Arrow. Arrow co-localises with Wingless in endosomes and is present in the basolateral domain where Wingless is stabilised by Dfz2. In this chapter I will investigate the possible contribution of Arrow to the degradation of Wingless bound to Dfz2.

4.2 Arrow brings down Wingless stabilised by Dfz2

In order to assess whether Arrow is able to bring about degradation of Wingless bound to Dfz2, I co-expressed Dfz2-FLAG and Arrow-HA in the Dpp domain. Under these conditions, Wingless is brought down to near wild-type levels (Figure 4.1a-a' and b-b''). The reduction of Wingless staining is not due to an effect of Arrow on the localisation of Dfz2 since Dfz2 levels seem unaffected under these conditions(compare Figure 4.1a' and b'). Dfz2 is observed to be present apically and basolaterally in the disc (Figure 4.1c'), Arrow is observed basolaterally (Figure 4.1c'').

Figure 4.1 Ectopic expression of Arrow represses Dfz2 induced stabilisation of Wingless

(a) Wing disc of the phenotype *dpp-gal4 UAS-Dfz2-FLAG* labelled with anti-Wingless (a) and anti-FLAG (a') shows stabilisation of Wingless.
(b) Wing disc of the phenotype *UAS-Dfz2-FLAG, dpp-gal4/UAS-Arrow-HA* labelled with anti-Wingless (b) anti-FLAG (b') and anti-HA (b''). Wingless is brought down to near wild-type levels indicating that in the presence of Arrow, it is no longer stabilised by Dfz2.
(c-c'') Optical cross-section of a wing disc expressing both Arrow and Dfz2. Labelled with anti-FLAG (c') and anti-HA (c''). Dfz2 is observed apically and basolaterally, Arrow is observed basolaterally.
(d-d') Ectopic expression of Arrow and Dfz2 leads to ectopic bristle formation, indicating a large increase in Wingless signalling, much greater than that of Dfz2 or Arrow alone (compare to 3.10b-b' and 3.11c-c').


a
Wg
a'
Fz2
b
Wg
b'
Fz2
b''
Arrow
c
Merge
c'
Fz2
c''
Arrow
d
Fz2+Arrow
d'
Fz2+Arrow

Reduction of the Dfz2-stabilised Wingless could be due to Arrow itself or to a downstream effect of increased signalling caused by co-expression of the two receptors. Indeed, co-expression of Arrow and Dfz2 leads to a large increase in Wingless signalling. When kept at 25^{0}C, flies expressing Dfz2 and Arrow in the Dpp domain do not hatch from the pupal case a likely effect of excess signalling. When kept at 21^{0}C, (in order to reduce the activity of gal-4), flies hatch and the wings exhibit multiple ectopic bristles (Figure 4.1d-d').

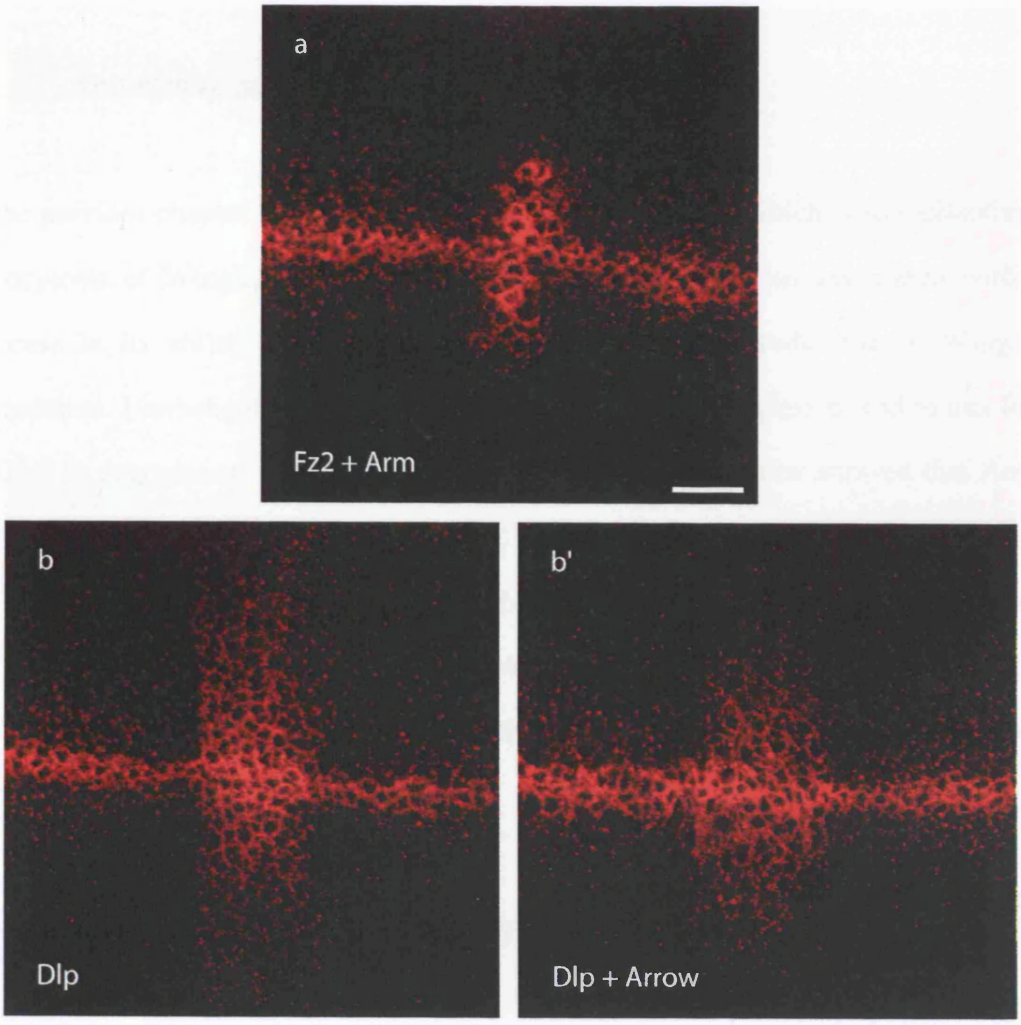
4.3 Arrow specifically acts on Dfz2 associated Wingless

The results described above suggest that Arrow has the ability to target Dfz2 bound Wingless to degradation. I then investigated whether Arrow has indiscriminate Wingless degrading activity or whether it specifically acts on Wingless associated with Dfz2. To address this question, I assessed the ability of Arrow to remove Wingless bound to Dally-like. Overexpression of Dally-like in the wing disc leads to Wingless stabilisation (Baeg et al., 2001)(Figure 4.2b). When both Arrow and Dally-like are overexpressed, a slight reduction in Wingless stabilisation is observed, suggesting Arrow is able to act on Wg stabilised by Dally-like (directly or indirectly). However, Wingless is still extensively stabilised (Figure 4.2b'). Indicating that Arrow is not able to act as efficiently on Wingless bound to Dally-like as it is on Wingless bound to Dfz2. It is possible that Arrow is unable to efficiently remove Wingless bound to Dally-like as Dally-like stabilises Wingless in a different domain to Dfz2, rendering it inaccessible to Arrow. However, Dally-like is present on the basolateral surface of imaginal disc cells (Kreuger et al., 2004) and stabilises Wg in the basolateral domain when overexpressed (X. Franch Marro personal communication), similarly to Dfz2. As Arrow is present on the basolateral surface and can remove Wingless stabilised basolaterally by Dfz2, it is unlikely that Arrow is unable to efficiently remove Wingless bound to

Dally-like due to the mislocalisation of the stabilised Wingless. This suggests that there is a specific cooperation between Arrow and Dfz2 that targets Wingless to degradation.

Figure 4.2 Specificity of Arrow's ability to degrade Wingless

(a) Wingless is stabilised by Dfz2 in the presence of activated Armadillo.
(*UAS-Arm [S10]; UAS-Dfz2-FLAG dpp-gal4*).
Discs derived from the progeny of *UAS-Arm [S10]/Y* x *Dpp-Gal4 UAS-Dfz2-FLAG/TM6B*. Female, non-TM6B larvae were selected and dissected.
(b) Ectopic expression of Dally-like stabilises Wingless (*dpp-gal4 UAS-dally-like*).
(b') Dally-like induced stabilisation of Wingless is not repressed by addition of Arrow (*UAS-Arrow-HA,dpp-gal4/UAS-dally-like*).
(b-b') Discs derived from the progeny of UAS-Arrow-HA/Y; dpp-Gal4/+ x UAS-Dally-like-HA. (b) male progeny from the cross and (b') female progeny.
All images are single confocal sections taken in the basolateral domain of the disc.


a
Fz2 + Arm
b
Dlp
b'
Dlp + Arrow

4.4 Arrow may contribute to Wingless endocytosis

In the previous chapter, I described a form of Dfz2, Dfz2AV, which is less effective at endocytosis of Wingless. This reduction in endocytosis is also associated with an increase in its ability to stabilise Wingless, suggesting a reduction in Wingless degradation. I investigated whether Arrow is able to direct Wingless bound to this form of Dfz2 to degradation. Co-expression of both Dfz2AV and Arrow showed that Arrow was indeed able to remove Wingless stabilised by Dfz2AV (Figure 4.3a-a' and b-b''). This suggests that in this situation, Arrow could be providing an internalisation signal. As shown previously, expression of Dfz2AV leads to ectopic activation of Wingless signalling (Figure 3.8b). Therefore the joint activity of Dfz2AV and can lead to both signalling and degradation.

4.5 Arrow is unable to degrade Wingless bound to Dfz2-GPI

As shown previously, Dfz2-GPI endocytoses Wingless very poorly (Figure 3.6 and Table 3.1). I investigated whether Arrow was able to degrade Wingless bound to this form of Dfz2. When both Arrow and Dfz2-GPI are expressed, Wingless is still stabilised (Figure 4.3c-c'), indicating that Arrow is unable to remove Wingless bound to Dfz2-GPI. This is unlikely to be due to the mislocalisation of Dfz2-GPI with respect to Arrow as Wingless is stabilised basolaterally by Dfz2-GPI (Figure 3.7) where Arrow is present and able to act on Dfz2 full-length. This suggests that the cooperation between Arrow and Dfz2 requires regions beyond the cysteine-rich domain in Dfz2 (which binds Wingless) in order for degradation to take place.

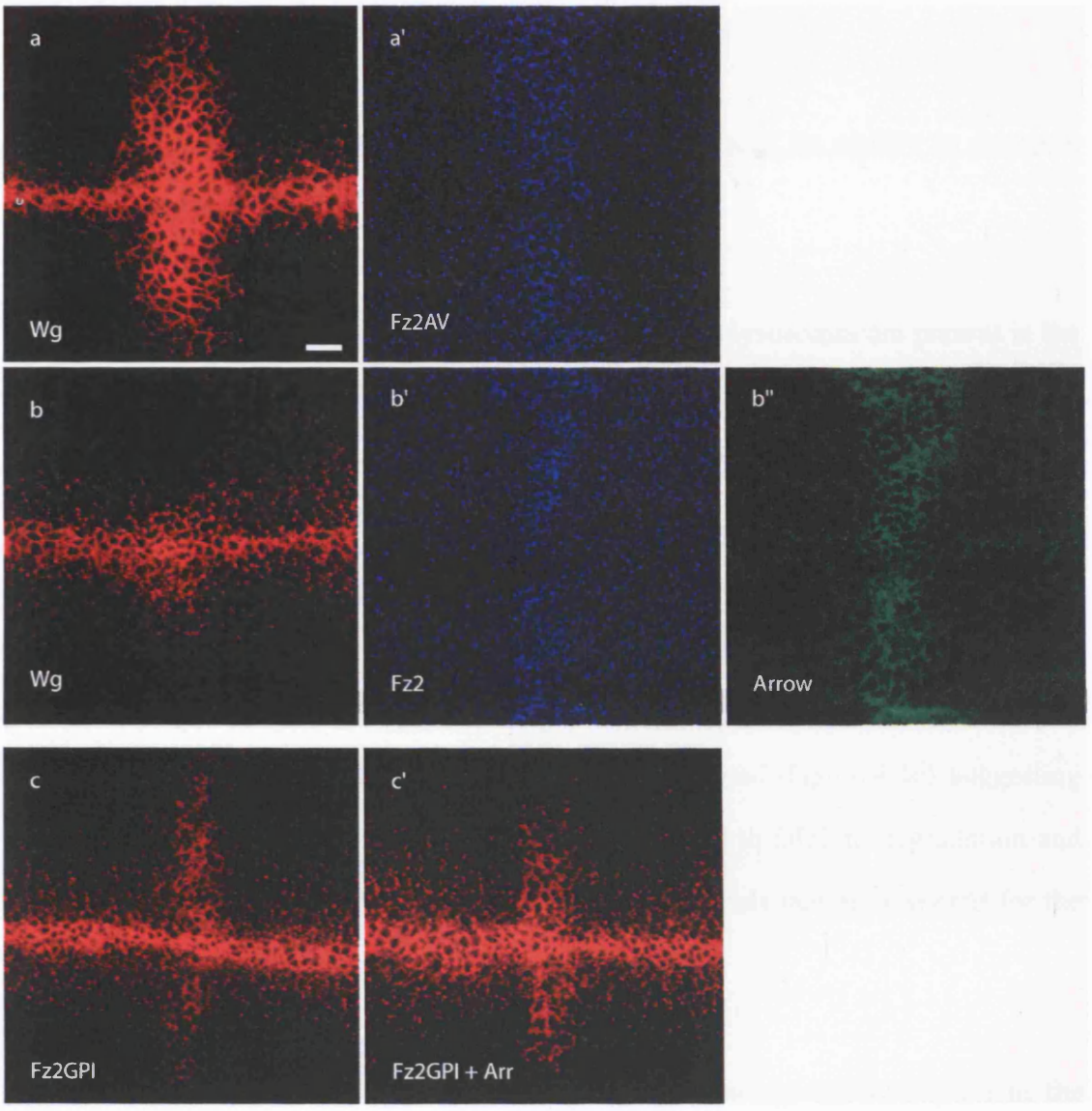
This result also further illustrates that Arrow does not have indiscriminate Wingless degrading activity, as Wingless bound by Dfz2-GPI cannot be efficiently degraded.

Figure 4.3 Arrow can degrade Wingless bound to Dfz2AV but not Wingless bound to Dfz2-GPI

(a-a') Wingless is stabilised by Dfz2AV. Wing disc of the genotype *dpp-gal4 UAS-Fz2AV* labelled with anti-Wingless (a) and anti-FLAG(a').

(b-b'') Wingless stabilisation by Dfz2-AV is repressed in the presence of Arrow. Wing disc of the genotype *dpp-gal4 UAS-Dfz2AV, UAS-Arrow-HA* labelled with anti-Wingless (b), anti-FLAG(b') and anti-HA (b'').

(c-c') Wingless is stabilised by Dfz2-GPI but this stabilisation is not suppressed by the expression of Arrow. Discs derived from the progeny of UAS-Arrow-HA/Y; dpp-Gal4/+ x UAS-Dfz2-GPI. (c) Male progeny from the cross and (c') female progeny.


a
Wg
a'
Fz2AV
b
Wg
b'
Fz2
b''
Arrow
c
Fz2GPI
c'
Fz2GPI + Arr

4.6 Truncating the cytoplasmic tail of Arrow blocks its ability to degrade Wingless

It is likely that any potential signals that target Wingless to lysosomes are present in the cytoplasmic tail of Arrow. In order to assess this possibility, I generated a form of Arrow that has the residues 1477-1612 from the cytoplasmic tail deleted (ArrowΔC) and is tagged with the HA-epitope (Figure 4.4a). Overexpression of this form of Arrow in the Wing disc results in mild stabilisation of Wingless (Figure 4.4b), which is not observed when Full-length Arrow is overexpressed (Compare to Figure 3.9a). This suggests that this form of Arrow could be less efficient at trafficking Wingless. When ArrowΔC is coexpressed with Dfz2, Wingless is still stabilised (Figure 4.4c), suggesting that ArrowΔC does not efficiently direct Wingless bound to Dfz2 to degradation and therefore, that the cytoplasmic tail of Arrow contains signals that are required for the trafficking of Wingless to lysosomes.

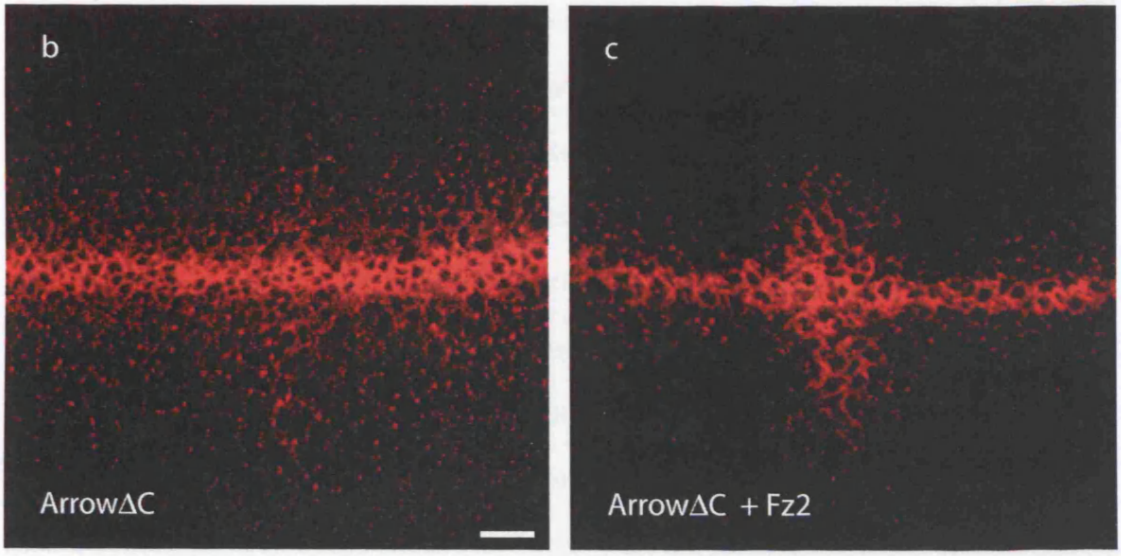
ArrowΔC does not appear to signal very efficiently. Adult wings of flies expressing the truncated form of Arrow do not differ from wild-type (Figure 4.4d), whereas expression of full-length Arrow leads to ectopic pathway activation (Figure 3.9b). Interestingly however, Expression of ArrowΔC using the ubiquitous Arm-Gal4 driver is able to rescue an *arrow* mutant fly to adulthood (data not shown). This suggests that overexpressed ArrowΔC is able to signal at a level sufficient to support development, but is not sufficient to activate ectopic signalling upon overexpression.

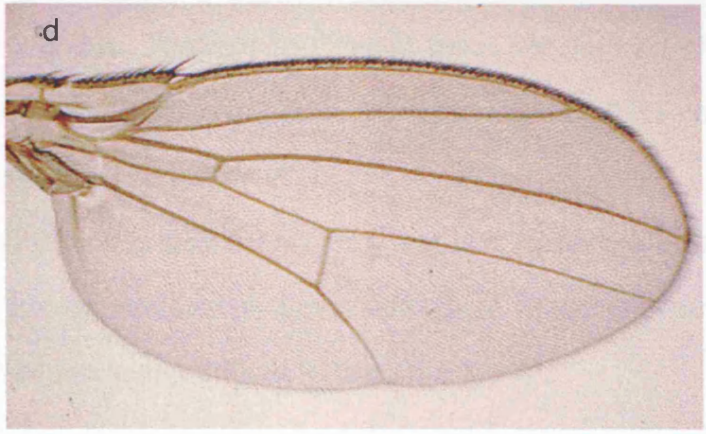
Figure 4.4 The Arrow cytoplasmic tail contains the degradation signal

(a) The Arrow cytoplasmic tail. Highlighted in red is the region deleted in ArrowΔC.
(b) Wing disc of the genotype *UAS- ArrowΔC; dpp-gal4*, Wingless is weakly stabilised in the presence of ArrowΔC (note that Wingless distribution is unaffected in the presence of full-length Arrow). Wing disc derived from *ArrowΔC* x *dppGal4/TM6B*, non-*TM6B* larvae selected.
(c) Wing disc of the genotype *UAS- ArrowΔC; dpp-gal4 UAS-Dfz2-FLAG*, Wingless is still stabilised by Dfz2 in the presence of ArrowΔC (compare to Fig.4.1a'). Wing disc derived from *UAS- ArrowΔC* x *dpp-gal4 UAS-Dfz2-FLAG/TM6B* non-*TM6B* larvae selected.
(d) Wing of the same genotype as in b, ectopic expression of ArrowΔC, does not lead to increased activation of the Wingless pathway, unlike ectopic expression of full-length Arrow (compare to Fig 3.9b).
Confocal images are single basolateral sections.

a

TRIGKSRTEPKDDQATDPLSPSTLSKSQRVSKIASVADAVRMSTL
NSRNSMNSYDRNHITGASSSTTNGSSMVAYPINPPPSPATRSRRP
YRHYKIINQPPPPTPCSTDICDESDSNYTSKSNSNNSNGGATKHS
SSS**AAACLQYGYDSEPYPPPPTPRSHYHSDVRIVPESSCPPSPSS**





4.7 Arrow mutant clones contain elevated levels of Wingless

Blocking lysosomal degradation in the wing disc leads to the accumulation of Wingless protein (L. Dubois personal communication). If, as the gain of function experiments suggest, Arrow is required for directing Wingless endocytosed by Dfz2 to lysosomes then in the absence of Arrow similar phenotypes should be observed. To investigate whether this was the case, I assessed Wingless distribution in wing discs that contained *arrow* mutant clones generated by mitotic recombination.

arrow mutant clones survive poorly (Wehrli et al., 2000) (and my own observations). Using hs-flp to generate the clones results in relatively few mutant cells being recovered as compared to their wild-type twins. Moreover, these clones tend to be small, making accurate assessment of the Wingless distribution difficult. To overcome this problem, I used a genetic system where flipase is driven with the *lama-gal4* driver that expresses throughout the wing disc. Furthermore, mutant tissue was given a growth advantage over the twin spots due to the presence of a cell lethal on the FRT chromosome (See materials and methods for further details).

When this genetic system is used, large clones that lack Arrow can be produced (Figure 4.5a and b) and these clones contain elevated levels of Wingless protein up to 10 cells away from the Wingless source (Figure 4.5a-a', b-b').

The increase in Wingless levels could be due to decreased degradation of Wingless or to increased transcription of Wingless in the mutant cells. Wingless refines its own expression domain by repressing transcription in the 2-3 cells adjacent to the Wingless expressing cells (Rulifson et al., 1996). As Wingless is observed at elevated levels up to 10 cells away from the D-V boundary in *arrow* mutant clones, it is unlikely that this effect is due to ectopic transcription. However, this possibility must be formally ruled out. To do this, *in situ* hybridisation was carried out on discs containing *arrow* mutant

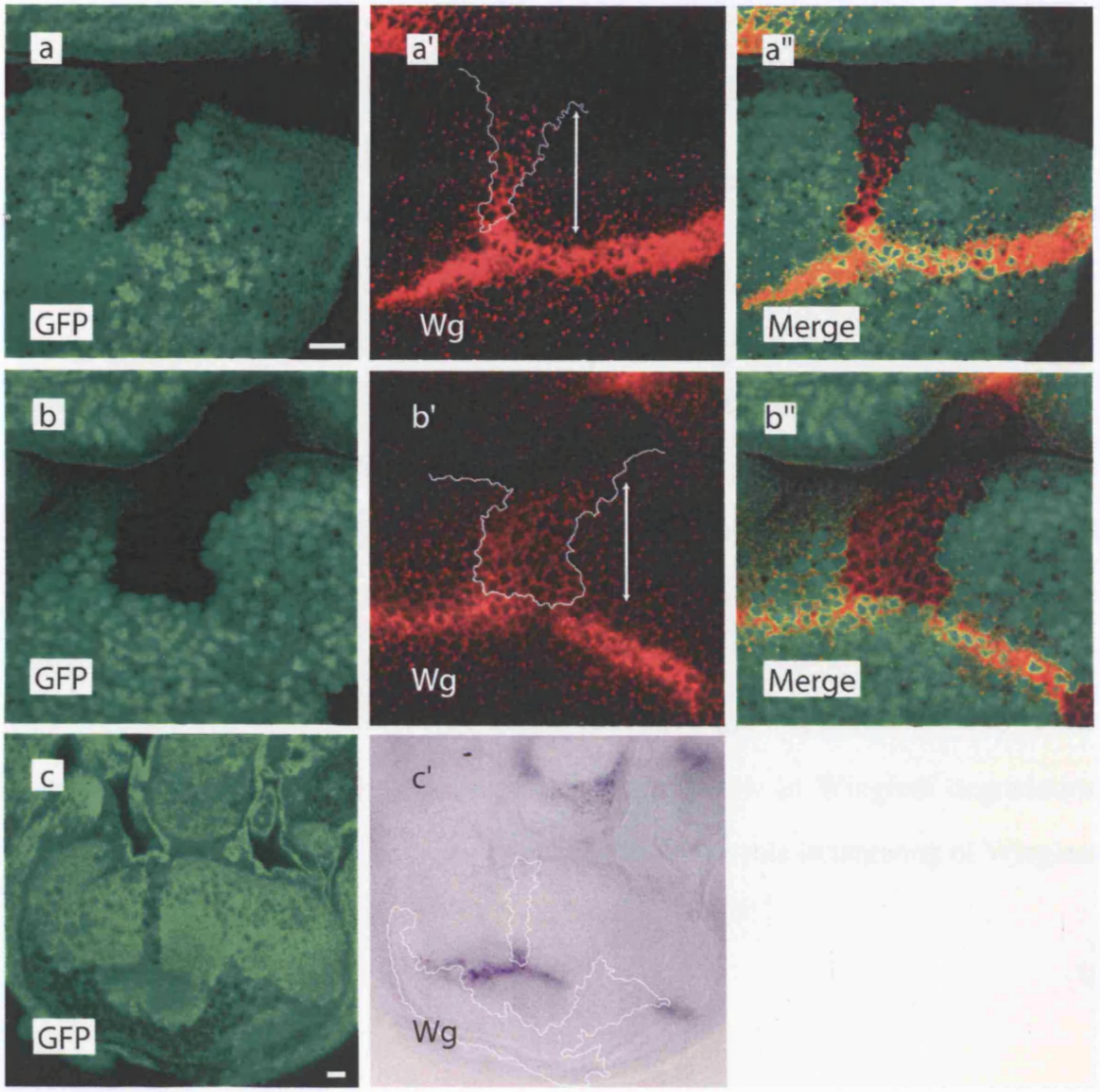
clones (Figure 4.5c-c'). This showed that as previously reported for Dsh clones, Wingless transcription is only activated in the 2-3 cells adjacent to the normal Wingless expressing cells. I conclude that increased Wingless transcription is not the reason why levels of Wingless protein accumulate in *arrow* mutant clones.

Figure 4.5 Cells lacking Arrow accumulate Wingless

(a-a'') and (b-b'') Homozygous *arrow* mutant clones marked by the absence of GFP(green) labelled with anti-Wingless (red) accumulate Wingless up to 10 cells away from the source of Wingless. Images are single confocal sections in the basolateral domain of the disc.

(c-c') *in situ* hybridisation of Wingless mRNA on discs containing *arrow* mutant clones. Clones are marked by the absence of GFP (c), Wingless mRNA in blue (c'). Wingless transcription is observed in the clone in the 2-3 cells closest to the wingless producing cells that are mutant for Arrow. However, beyond wingless is not transcribed.

Genotype in all images: *FRT42D pcna/FRT42D pwn* arr^2*; lama-gal4 UAS-flp.*


a
GFP
a'
Wg
a''
Merge
b
GFP
b'
Wg
b''
Merge
c
GFP
c'
Wg

4.8 Wingless can be internalised in the absence of Arrow

As previously suggested, Arrow may contribute to Wingless endocytosis (Figure 4.3a and b). The accumulated Wingless observed in Arrow mutant clones might therefore be due to a requirement for Arrow in Wingless endocytosis. In order to assess whether this is the case, I investigated whether Wingless is internalised in the absence of Arrow. Discs containing *arrow* mutant clones were labelled with fluorescent dextran to visualise the endocytic compartment as previously described. Within *arrow* mutant clones Wingless is present at the cell surface and also in endocytic structures (Figure 4.6a-a''').

This suggests that while Arrow might contribute to Wingless endocytosis, it is not essential for it to take place. The requirement for Arrow in Wingless degradation therefore appears to be after endocytosis, consistent with a role in targeting of Wingless internalised by Dfz2 to lysosomes.

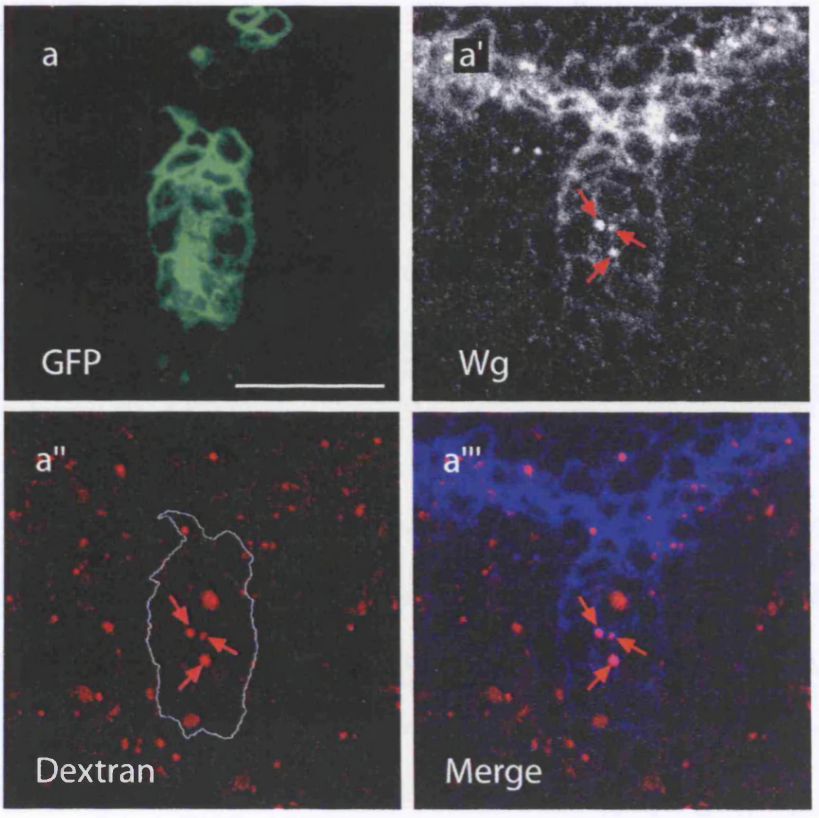


Figure 4.6 Wingless is internalised in the absence of Arrow

Homozygous arrow mutant clones of cells marked by the presence of GFP (a), labelled with anti-Wingless (a') and Texas Red dextran (a'') as previously described. Merge of (a') and (a'') in (a'''). Accumulated Wingless is observed at both the cell surface and in dextran-positive vesicles (Arrows). Images are single confocal sections in the basolateral domain of the disc.

Genotype: *hs-flp, UAS-CD8-GFP; FRT^{42D} pwn arr^{2} / FRT^{42D} Tub-gal80; Tub-gal4*

4.9 Wingless Distribution is unaffected in *fz, Dfz2* clones

The requirement for Arrow in degradation leads to the accumulation of Wingless in the cells that lack Arrow (Figure 4.5). As my previous work implicates Dfz2 in the pathway that leads to degradation of Wingless, I assessed whether removal of Dfz2 resulted in similar phenotypes to that of removal of Arrow. As Fz and Dfz2 can act redundantly in Wingless signalling, it is possible that in the absence of Dfz2, Fz could compensate for its functions. Therefore, I assessed Wingless distribution in cells that lack both Fz and Dfz2.

In *fz, Dfz2* clones, Wingless levels are elevated in the 2-3 cells closest to the Wingless expressing cells at the D-V boundary (Figure 4.7). However, further from the Wingless source, no effect on Wingless distribution is observed. The increase in Wingless levels close to the source are a likely result of increased Wingless transcription, as observed in cells that lack Dishevelled (Rulifson et al., 1996) and Arrow (Figure 4.5 c-c').

The work I have previously described suggests that by virtue of its ability to internalise Wingless, Dfz2 contributes to degradation of Wingless. It might be expected that removal of Dfz2 would lead to accumulation of Wingless, however, this is not the case. A possible explanation for this is that due to the requirement for Fz and Dfz2 in Wingless binding, cells that lack Fz and Dfz2 cannot efficiently capture Wingless, which would mask any effect on Wingless degradation caused by the absence of Dfz2.

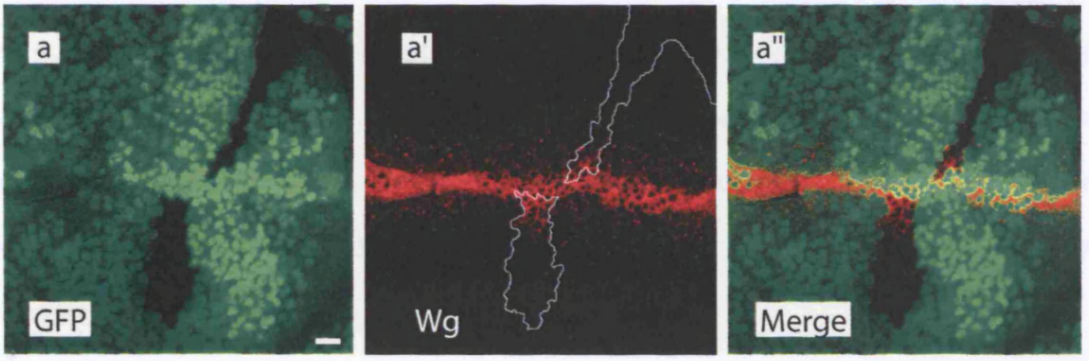


Figure 4.7 Wingless distribution in *fz, Dfz2* mutant clones

Homozygous mutant clones of *fz* and *Dfz2* marked by the absence of GFP (a) exhibit an increase in Wingless protein levels in the 1-2 cells closest to the normal source of Wingless (a'-a''). However, further from the source, Wingless distribution in the clone is unaffected. The increase in protein levels is the likely result of ectopic Wingless transcription in the absence of Wingless signalling, as observed in Figure 4.5c-c' Images are single confocal sections in the basolateral domain.

Genotype: *hs-flp; fz [H51], Dfz2 [C1] ri FRT2A/FRT2A Ubi-GFP*

4.10 *dishevelled* clones accumulate Wingless

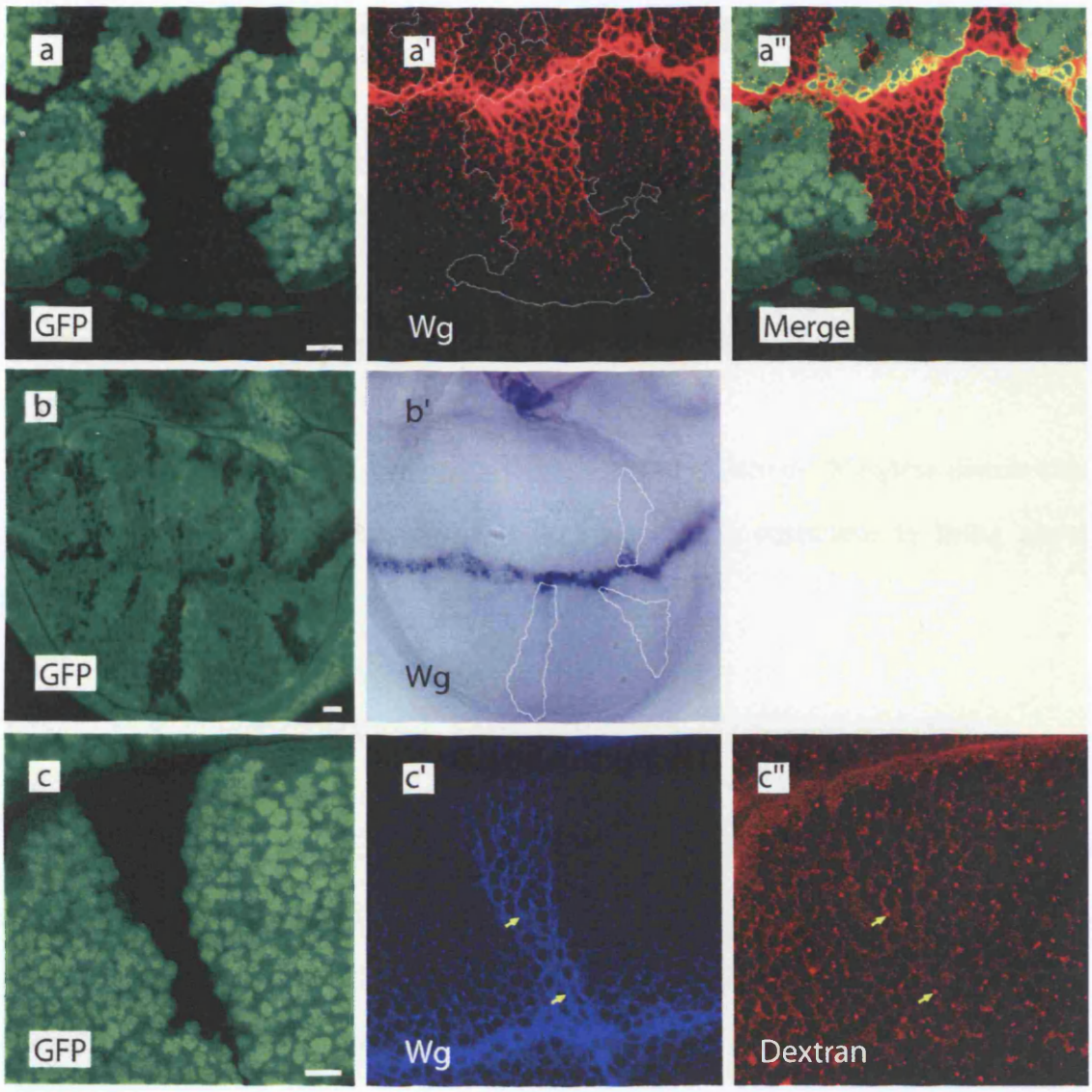
The downstream components that associate with the Dfz2-Wingless complex may be involved in the endocytosis and degradation of Wingless. Indeed, in human embryonic kidney cells, upon stimulation by Wnt5a, Dsh mediates endocytosis of Frizzled-4 (Chen et al., 2003). It is possible that in *Drosophila*, Dsh mediates Dfz2 endocytosis and consequently, Wingless internalisation. I investigated whether Dsh has a role in Wingless endocytosis and degradation.

First, I investigated the effect that removal of Dsh has on Wingless distribution by making mitotic clones of *dsh* in the wing disc. Cells that lack Dsh show increased levels of Wingless (Figure 4.8a-a'') in a similar manner to removal of Arrow (Figures 4.5a and b). This is consistent with a potential role of Dsh in Wingless trafficking. In order to confirm that the elevated levels of Wingless protein do not arise from increased transcription, I assessed *wingless* transcription in the *dsh* mutant clones. As with *arrow*, and as shown previously for *dsh* (Rulifson et al., 1996), *dsh* mutant cells upregulate *wingless* transcription only in the 2-3 cells closest to the normal Wingless expressing cells (4.8b-b'). This is not likely to be sufficient to account for the increase in Wingless levels observed in *dsh* mutant clones and suggests that Dsh may indeed be a component of the complex that degrades Wingless.

If Dsh were essential for internalisation of Dfz2, and consequently Wingless, it would be expected that removal of Dsh might block Wingless endocytosis. To assess this possibility, I carried out an endocytosis assay using dextran on discs containing *dsh* mutant clones (Figure 4.8c-c''). Wingless is still internalised in the absence of Dsh (arrows in Figure 4.8c'-c''), suggesting that removal of Dsh is not essential for Wingless endocytosis.

Figure 4.8 Cells lacking Dsh accumulate Wingless but Dsh is not required for Wingless internalisation

(a-a'') Homozygous *dsh* mutant clones of cells marked by the absence of GFP labelled with anti-Wingless (red) accumulate Wingless up to 15 cells away from the source of Wingless. Images are basolateral single confocal sections.
(b-b') *in situ* hybridisation of Wingless mRNA on discs containing *dsh* mutant clones. Clones are marked by the absence of GFP (b), Wingless mRNA in blue (b'). Wingless transcription is observed in the 2 cells closest to the Wingless producing cells that are *dsh* mutant. However, further away Wingless is not transcribed.
(c-c') *dsh* mutant clones labelled by the absence of GFP (c) accumulate Wingless (c') at the cell surface and in endocytic structures labelled by dextran Texas-red (c''). Images are single confocal sections. Images are basolateral single confocal sections.


a
GFP
a'
Wg
a''
Merge
b
GFP
b'
Wg
c
GFP
c'
Wg
c''
Dextran

4.11 Dsh it not sufficient to bring down Wingless bound to Dfz2

While Dsh does not appear to be essential for Wingless endocytosis, the possibility still remains that Dsh mediated internalisation of Dfz2 and Wingless could be the mechanism by which degradation of Wingless occurs. I therefore assessed whether ectopic expression of Dsh is able to bring down Wingless stabilised by Fz2, as had been observed for Arrow.

When both Dsh and Fz2 are expressed using the *dpp-gal4* driver, Wingless distribution is unaffected (Figure 4.9), suggesting that Dsh is not sufficient to bring about degradation of Wingless bound to Dfz2.

4.12 Extracellular Wingless levels are elevated in the absence of Arrow

Wingless forms an extracellular gradient on the basolateral surface of the imaginal disc epithelium (Strigini and Cohen, 2000). As I have previously described, using conventional antibody labelling techniques, Wingless accumulates in the absence of Arrow. I investigated whether in the absence of Arrow, the extracellular gradient of Wingless is affected. Extracellular Wingless distribution was examined in discs containing *arrow* mutant clones using the extracellular staining technique of Strigini and Cohen (2000). In *arrow* mutant clones, extracellular Wingless accumulates up to 10 cells away from the Wingless source (Figure 4.10a-a'). This suggests that degradation by Arrow shapes the extracellular Wingless gradient. Removal of *fz* and *Dfz2* leads to an increase in Wingless close to the source (Figure 4.10b-b'), the likely result of increased transcription of Wingless in these cells. However, as observed with the total staining (Figure 4.6), the extracellular Wingless distribution is unaffected further away from the D-V boundary.

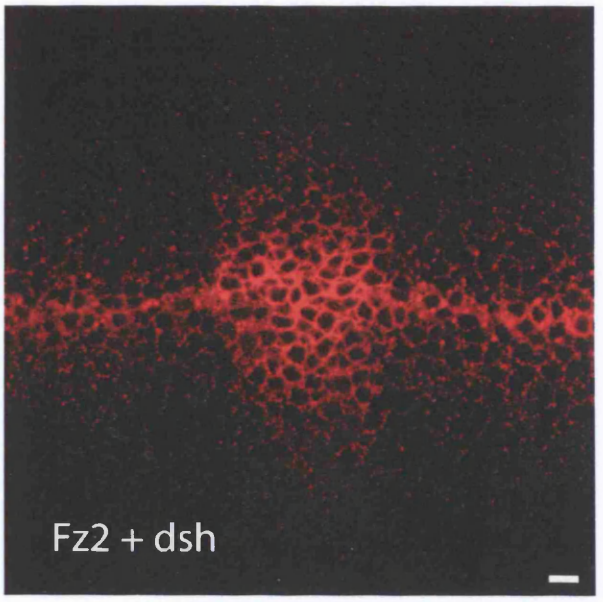
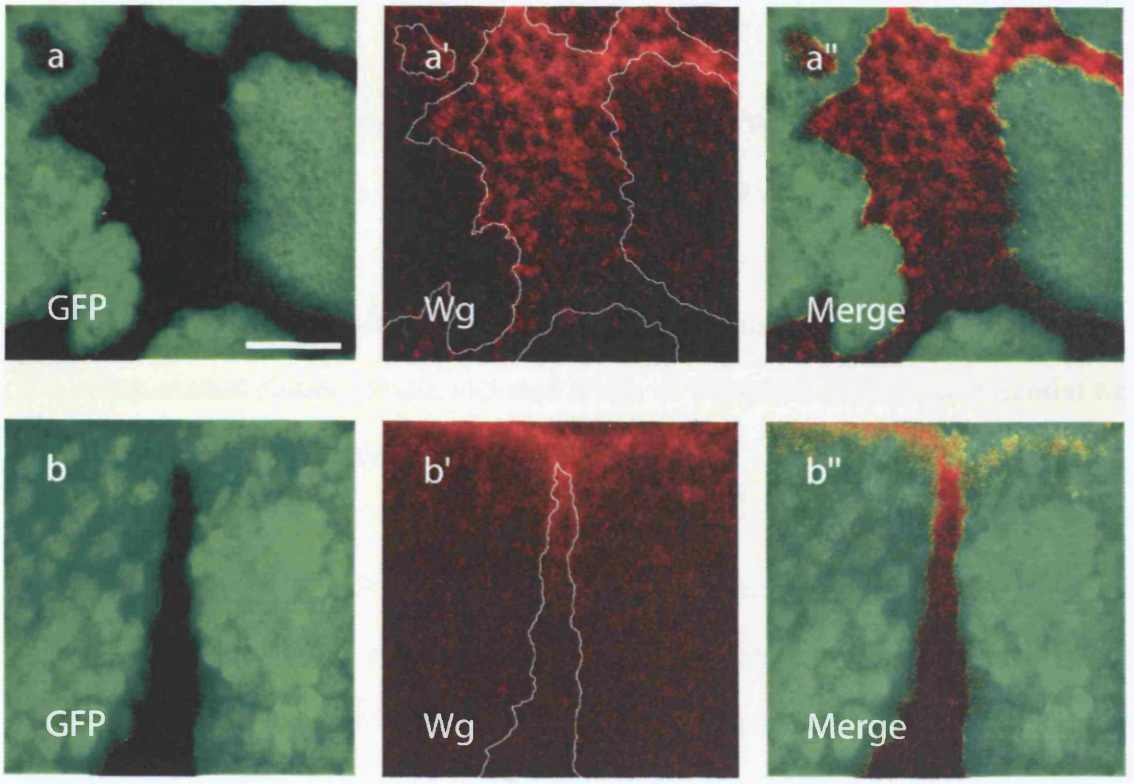


Figure 4.9 Ectopic expression of Dsh does not affect Wingless stabilised by Dfz2

Wing disc of the genotype *UAS-dsh; dpp-gal4 UAS-Dfz2-FLAG* labelled with anti-Wingless. Wingless is still stabilised by Dfz2. Wing disc derived from a cross of *UAS-Dsh/TM6B* x *dpp-gal4 UAS-Dfz2-FLAG/TM6B* non-TM6B larvae were selected. All images are basolateral single confocal sections.

Figure 4.10 *arrow* mutant clones accumulate extracellular Wingless while *fz, Dfz2* clones do not

(a-a'') Homozygous *arrow* mutant clones of cells marked by the absence of GFP labelled with anti-Wingless (red) accumulate extracellular Wingless up to 10 cells away from the source of Wingless.
(b-b'') Homozygous *fz Dfz2* mutant clones of cells marked by the absence of GFP labelled with anti-Wingless (red) accumulate Wingless in the 2 cells closest to the D-V boundary but further away, extracellular Wingless distribution is unaffected.
All images are single confocal sections.
Genotypes:
(a-a') *FRT42D pcna/FRT42D pwn arr [2], lama-gal4 UAS-flp.*
(b-b') *hs-flp, fz [H51], Dfz2 [C1] ri FRT2A/FRT2A Ubi-GFP.*


a
GFP
a'
Wg
a''
Merge
b
GFP
b'
Wg
b''
Merge

4.13 Summary

- Expression of Arrow brings down Wingless stabilised by Dfz2.
- Arrow is unable to efficiently bring down Wingless bound to Dally-like or Dfz2-GPI.
- Truncating the cytoplasmic tail of Arrow prevents Wingless degradation.
- *arrow* mutant clones contain elevated levels of Wingless while *fz, Dfz2* clones do not.
- Wingless can be endocytosed in the absence of Arrow.
- Dsh mutant clones contain elevated levels of Wingless. Dsh is not essential for Wingless internalisation.

The work in the Chapter 3 illustrates that Dfz2 is involved in the endocytosis of Wingless. However, this is not sufficient to lead to degradation as overexpression of Dfz2 stabilises Wingless. I speculated that under these experimental conditions, a factor might become limiting, preventing degradation of Wingless after endocytosis by Dfz2. The work in this chapter indicates that this is indeed the case and that Arrow is the limiting factor. Overexpression of Arrow brings down Wingless stabilised by Dfz2 and it appears that a signal that enables it to do this could reside in the cytoplasmic tail of Arrow, as deleting part of it impairs its degradative activity. Removal of Arrow causes accumulation of Wingless, which further implicates it in Wingless degradation. Interestingly, it appears that in addition to Dfz2, Arrow can also provide an endocytic signal, as it is able to degrade Wingless bound to a form of Dfz2 that is inefficient at Wingless internalisation.

Arrow cannot efficiently degrade Wingless bound to Dally-like and also cannot degrade Wingless bound to Dfz2-GPI. Therefore, the cooperation between Arrow and Dfz2 is specific and requires parts of Dfz2 beyond the extracellular cysteine-rich domain that binds Wingless.

While Dfz2 is obviously part of the machinery that brings about degradation, *fz, Dfz2* clones do not accumulate Wingless, this is possibly due to reduced Wingless-capturing activity of the cells that lack Fz and Dfz2.

Together these findings suggest a mechanism for Wingless degradation that involves firstly, capture by Dfz2, followed by internalisation stimulated by Dfz2 and possibly Arrow, and finally targeting to lysosomes by Arrow.

The role of Dsh in this mechanism remains somewhat unclear; cells that lack Dsh accumulate Wingless, suggesting a possible role in Wingless degradation. As previously described Dsh has been implicated in the endocytosis of Frizzled proteins (Chen et al., 2003), however I find that Wingless is endocytosed in the absence of Dsh. Suggesting either that alternative mechanisms of Wingless endocytosis exist or that Dsh is not essential for Dfz2 endocytosis in fly cells. Further investigation of the role of Dsh is necessary before reaching a conclusion as to its function in Wingless trafficking.

CHAPTER 5 - The role of Arrow modifications in Wingless degradation

5 CHAPTER 5 - THE ROLE OF ARROW MODIFICATIONS IN WINGLESS DEGRADATION

5.1 Introduction

Various motifs have been identified that mediate receptor internalisation and degradation. These motifs are often associated with post translational modifications of the receptor. Two modifications that could regulate Arrow internalisation and degradation are phosphorylation and ubiquitination.

5.1.1 Phosphorylation

Phosphorylation of GPCRs is often followed by endocytosis (Lefkowitz, 1998). Phosphorylation leads to the recruitment of β-Arrestins. These bind with clathrin and AP-2 resulting in the recruitment into clathrin-coated pits (Luttrell and Lefkowitz, 2002). Phosphorylation also mediates the endocytosis of members of the LDL receptor family. LRP1 is a multifunctional receptor that mediates the uptake of lipoproteins, proteases and protease inhibitors (Nykjaer and Willnow, 2002). The cytoplasmic tail of LRP1 is phosphorylated by Protein kinase A (PKA) and this phosphorylation contributes to receptor-mediated endocytosis (Li et al., 2001). Blocking phosphorylation of the cytoplasmic tail of LRP1 reduces endocytosis and reduces degradation of the LRP1 ligand RAP (Li et al., 2001), indicating that phosphorylation of LRP1 regulates LRP and LRP ligand activity by degradation.

In order to further characterise the mechanism that leads to the targeting of Wingless to lysosomes by Arrow, I investigated whether phosphorylation of Arrow was required for Arrow to target Wingless to degradation. LRP6 is phosphorylated at five PPPSP motifs, which are conserved in LRP5 and Arrow (Tamai et al., 2004). This phosphorylation is necessary and sufficient to activate signalling in *Xenopus* embryos. When a modified

form of LRP6 (which has the extracellular domain deleted and only a single PPPSP motif) left on the intracellular side, it acts as a dominant activator of the pathway. Changing the PPPSP to PPPAP renders this molecule inactive (Tamai et al., 2004).
The phosphorylation or LRP6 creates a docking site for Axin (Tamai et al., 2004). The association of Axin with Arrow is likely to prevent formation of the inhibitory complex that degrades Armadillo. This would result in the stabilisation of Armadillo and its translocation to the nucleus where it can activate signalling.

5.1.2 Preventing Arrow phosphorylation reduces Wingless degradation

In order to assess the role of Arrow phosphorylation in Wingless trafficking, I generated a modified form of Arrow where the five PPP(S/T)P motifs are mutated to PPPAP (hereby referred to as Arrow-PPAP) (Fig 5.1a). Note that this is in the context of full-length Arrow, in contrast to the constructs used by Tamai et al (2004) which, in addition to the PPPAP mutations, had the extracellular domain deleted. This is important as I sought to maintain any possible interactions with Wingless and Dfz2. This construct was also tagged with the HA-epitope and introduced in flies.
I expressed this form of Arrow using the *dpp-gal4* driver and assessed its effects on Wingless distribution. Arrow-PPAP is expressed and causes a mild increase in Wingless protein levels in the domain of overexpression (Figure 5.1b-b'). This effect could be due to reduced Wingless degradation in the stripe where Arrow-PPAP is present, or alternatively, Arrow-PPAP could be acting as a dominant-negative with regards to Wingless signalling. This could result in increased transcription of Wingless in these cells. This remains an open question that will be addressed in the future.
In order to assess whether Arrow-PPAP can bring down Wingless bound to Dfz2, I co-expressed Dfz2-FLAG with Arrow-PPAP using the *dpp-gal4* driver. Figure 5.1c-c' shows that Wingless continues to be stabilised in the domain of overexpression. This suggests that removal of the phosphorylatable residues in Arrow prevents it from

directing Wingless, bound to Dfz2, to degradation. Since LRP6 is phosphorylated in response to Wnt (Tamai et al., 2004), it is likely that Arrow becomes degradation competent upon activation of signalling by Wingless. Preventing Arrow phosphorylation does not appear to affect localisation of Arrow as it is observed to localise to the basolateral surface of the imaginal disc cells in a similar manner to Arrow full-length (Figured-d'') and colocalises with Dfz2 in this domain.

Figure 5.1 An unphosphorylatable form of Arrow does not efficiently degrade Wingless

(a) The 5 PPP(S/T)P motifs present in the cytoplasmic tail of Arrow (highlighted in red), to make Arrow-PPAP, each Serine or Threonine residue was mutated to an Alanine.

(b-b') Wing disc of the genotype *dpp-gal4 UAS-Arrow-PPAP* stained with anti-HAII (b) and anti-Wingless (b'). Wingless is weakly stabilised in the region of overexpression.

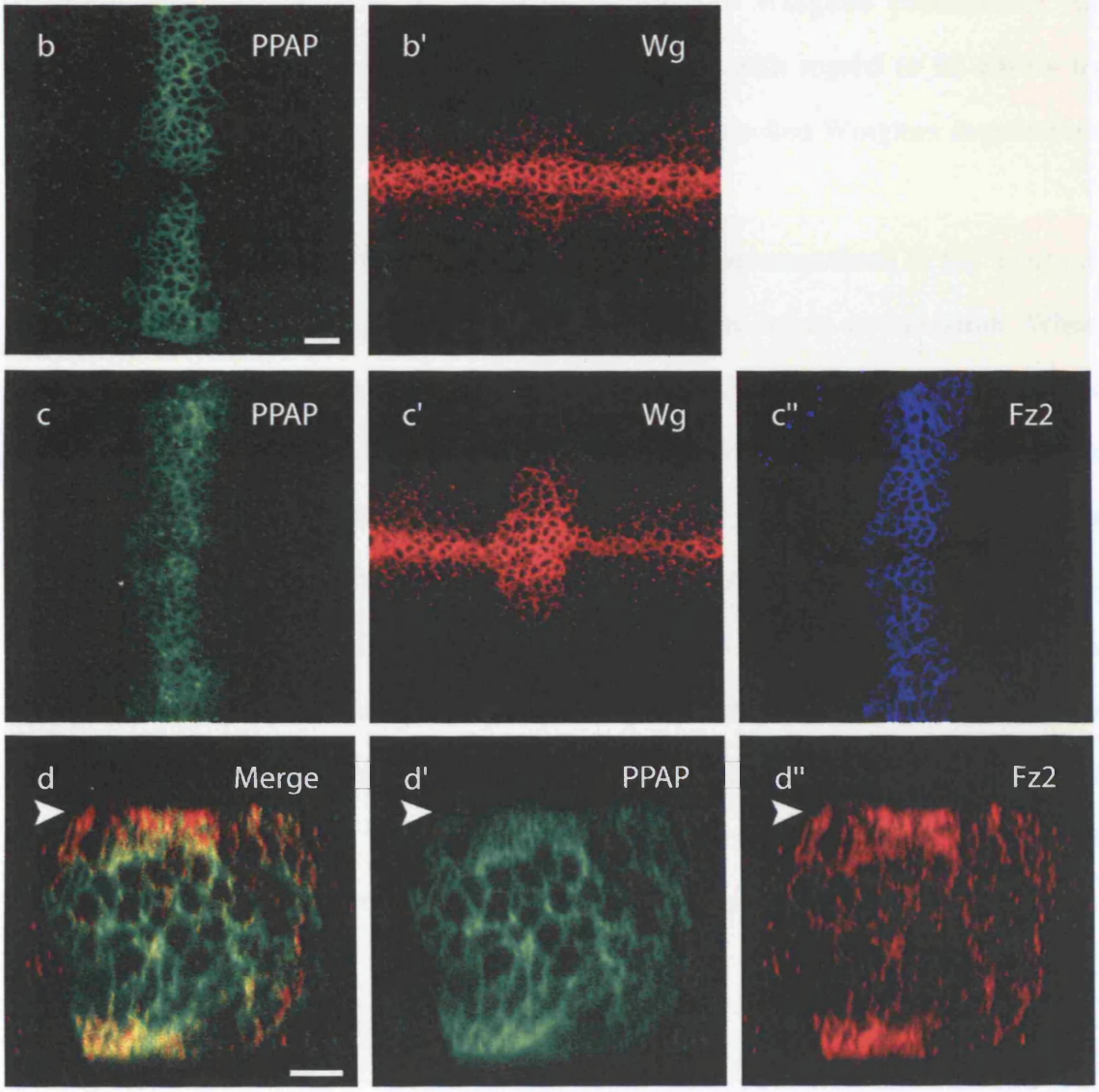
(c-c'') Wing disc of the genotype *dpp-gal4 UAS-Dfz2-FLAG UAS-Arrow-PPAP* labelled with antiHAII (b), anti-Wingless (c') and anti FLAG (c''). Wingless is stabilised by Dfz2, even in the presence of Arrow-PPAP.

(d-d'') Wing disc of the same genotype as (c-c'') labelled with anti-HA (green (d and d')) and anti-FLAG (red (d and d'')) Arrow-PPAP is primarily present in the basolateral domain where it colocalises with Dfz2, which is present in both the apical and basolateral domains.

All images are basolateral single confocal sections.

a

TRIGKSRTEPKDDQATDPLSPSTLSKSQRVSKIASVADAVRMS
TLNSRNSMNSYDRNHITGASSSTTNGSSMVAYPIN**PPPSP**ATR
SRRPYRHYKIINQP**PPPTP**CSTDICDESDSNYTSKSNSNNSNG
GATKHSSSSAAACLQYGYDSEPY**PPPPTP**RSHYHSDVRIVPES
SC**PPSP**SSRSSTYFSPL**PPPPSP**VQSPSRGFTZHFESYMZ



5.1.3 Arrow-PPAP colocalises with Wingless in Endosomes

My previous work has shown that Arrow colocalises with Wingless in endocytic structures and suggested that Arrow may contribute to Wingless endocytosis. As Arrow-PPAP behaves differently to Arrow full-length with regard to its ability to degrade Wingless, I investigated whether Arrow-PPAP blocked Wingless degradation by blocking Wingless endocytosis.

As described in Chapter 3, when full-length Arrow is over-expressed in the apterous domain, 52% of the punctate Wingless structures contain Arrow and Dextran. When Arrow-PPAP is expressed using *ap-gal4*, an increase in Wingless levels in the domain of overexpression is observed (Figure 5.2a-a'), as was observed when Arrow-PPAP was expressed using *dpp-gal4* (Figure5.1b'). The dextran labelling indicates that Wingless is present in endosomes and that these also contain Arrow-PPAP (Figure 5.2b-b'''). Quantification of this data showed that of the Wingless-positive punctate structures (within the domain of over-expression), 48% contain Arrow-PPAP and dextran. This is comparable to what is observed with full-length Arrow (Table 5.1). This suggests that Arrow-PPAP does not block Wingless degradation by blocking its endocytosis, and that phosphorylation of Arrow is not required for its internalisation.

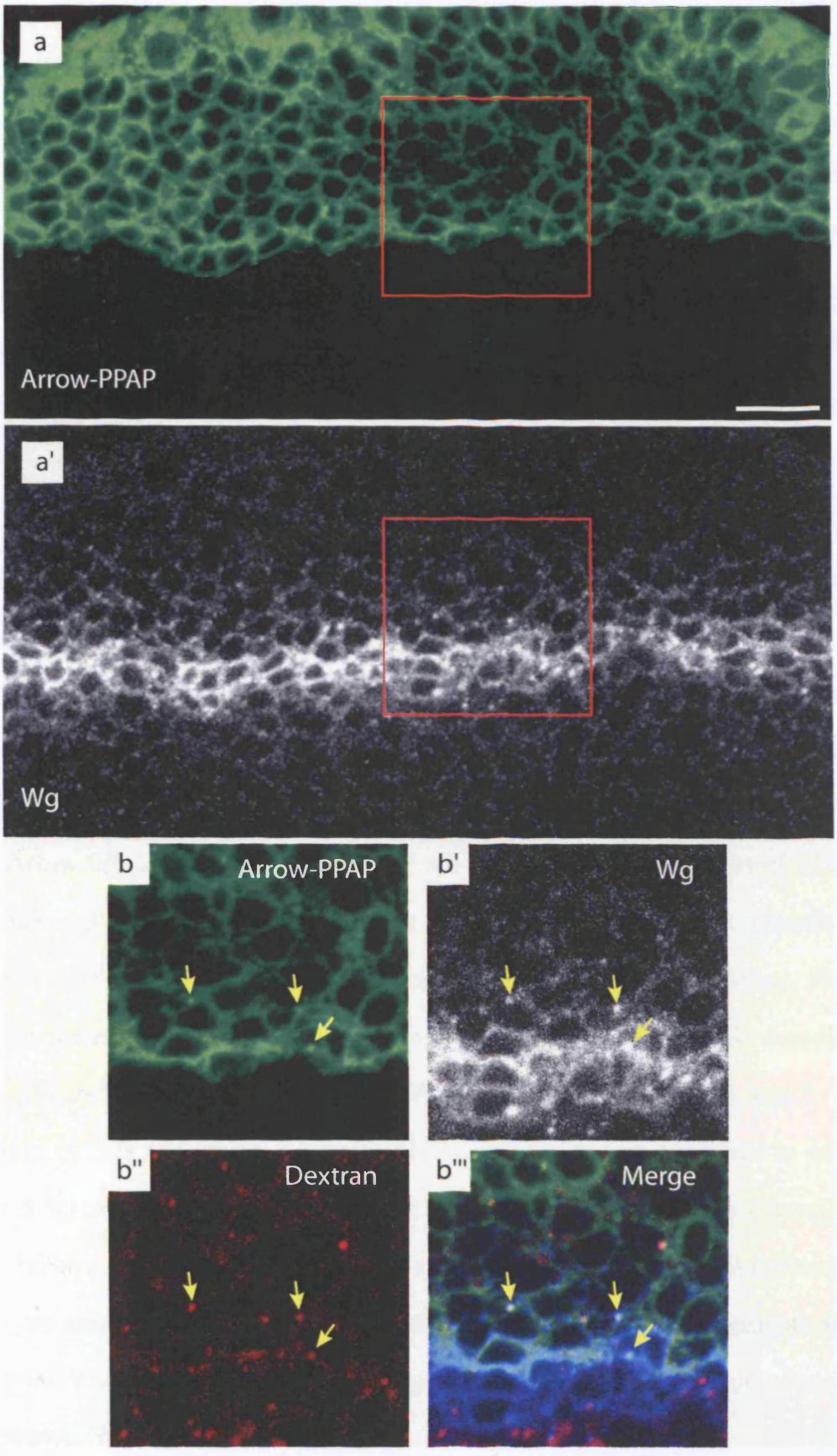
Figure 5.2 Blocking Arrow phosphorylation does not prevent Wingless internalisation

Wing disc of the phenotype *ap-gal4 UAS-Arrow-PPAP*, labelled with dextran-Texas Red and stained for anti-Wingless (white) and anti-HA (green).

(a-a') Punctate Wingless structures are observed in the domain of Arrow-PPAP expression.

(b-b''') High magnification image of the boxed region in (a-a') Punctate Wingless-positive structures colocalise with dextran and Arrow-PPAP (Arrows).

Images are basolateral single confocal sections.


a
Arrow-PPAP
a'
Wg
b
Arrow-PPAP
b'
Wg
b''
Dextran
b'''
Merge

5.1.4 Arrow-PPAP reduces Wingless signalling

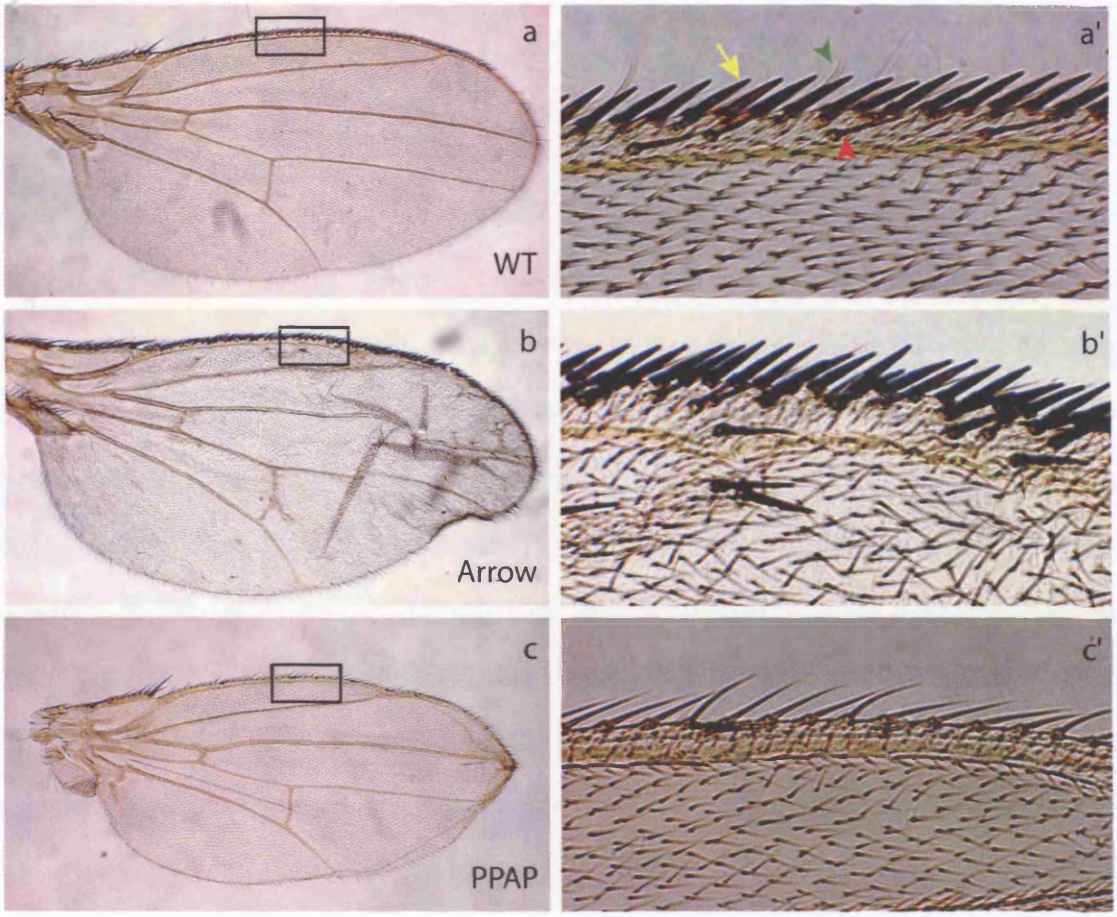
Previous work has indicated that phosphorylation of the PPP(S/T)P motifs is required for the activation of Wingless signalling (Tamai et al., 2004). I assessed the effect of ectopic expression of Arrow-PPAP on Wingless signalling by analysing the phenotype of adult wings. Ectopic expression of Arrow full-length using *dpp-gal4*, leads to increased Wingless signalling (Figure 3.9b-b'). By contrast, when Arrow-PPAP is expressed using *dpp-gal4*, no phenotype in adult wings was observed (data not shown). It was possible that a subtle effect of Wingless signalling was being missed due to the narrow range of expression of Dpp. Therefore, I studied the wings of adult flies where Arrow full length or Arrow-PPAP are expressed using *ap-Gal4* (a stronger a broader driver).

At the anterior wing margin of wild type wings, three rows of bristles are observed, a ventral row of slender bristles interspersed with chemosensory bristles, a row of stout bristles and a dorsal row of chemosensory bristles (Couso et al., 1994) and Figure 5.3a'. When Arrow full length and Arrow-PPAP are expressed under the control of the *ap-gal4* driver and kept at 25^{0}C the flies do not emerge from the pupal case. Therefore flies were kept at 21^{0}C, In order to reduce the activity of gal-4 (Phelps and Brand, 1998). At 21^{0}C, the wings of flies that express Arrow full-length show multiple ectopic stout bristles (Figure 5.3b-b') indicating ectopic Wingless signalling (Figure 5.3a-a').

The wings of flies expressing Arrow-PPAP are reduced in size compared to wild-type (Figure 5.3c) and exhibit a loss of margin bristles (Figure 5.3c'). The ventral row of slender bristles are still present but both the stout bristles and the dorsal chemosensory bristles are absent. This indicates that Arrow-PPAP is acting in a dominant-negative manner on Wingless signalling and suggests that indeed, Arrow phosphorylation participates in Wingless signalling.

Figure 5.3 Ectopic expression of Arrow-PPAP reduces Wingless signalling

(a-a') Dorsal view of a wild-type wing, boxed area in (a) shown in high magnification in (a'). Ventral slender bristles (green arrowhead), stout bristles (yellow arrow) and dorsal chemosensory bristles (red arrowhead) are indicated (Couso et al., 1994).
(b-b') Dorsal view of a wing of a fly of the genotype *ap-gal4 UAS-Arrow-HA* kept at 21°C. Ectopic stout bristles are observed, indicating ectopic Wingless signalling.
(c-c') Dorsal view of a wing of a fly of the genotype *ap-gal4 UAS-Arrow-PPAP* kept at 21°C. Note the reduced size of the wing and the loss of the stout bristles and the dorsal chemosensory bristles, indicating reduced Wingless signalling. The ventral slender bristles are still observed.


a
WT
a'
b
Arrow
b'
c
PPAP
c'

5.2 Ubiquitination

5.2.1 Introduction

Ubiquitination (also referred to as ubiquitylation) is a well-characterised protein modification. Ubiquitin is a 76 amino acid protein that is highly conserved throughout eukaryotes. The process of ubiquitination results in the covalent addition of ubiquitin to lysine residues in proteins. This process is carried out in a number of steps. Firstly an E1 ubiquitin-activating enzyme activates free ubiquitin, the activated ubiquitin is then transferred onto an E2 ubiquitin-conjugating enzyme, which in turn associates with an E3, ubiquitin ligase. E3 ubiquitin ligases catalyse the addition of ubiquitin to free lysine residues in the target protein (Weissman, 2001). The best understood function of ubiquitination results in the addition of multiple ubiquitin molecules in a chain, which earmarks proteins for degradation by the proteasome. However, ubiquitination also triggers the endocytosis of various cell surface proteins (Shtiegman and Yarden, 2003). Many examples of ubiquitination regulating the internalisation of cell surface proteins have been observed in yeast. The mating pheromone α-factor receptor, Ste2p of *Saccharomyces cerevisiae* undergoes ligand-induced endocytosis that is dependent on ubiquitination of the cytoplasmic tail(Hicke and Riezman, 1996). In this case the addition of a single ubiquitin molecule is sufficient to mediate internalisation, in contrast to the ubiquitin-mediated proteasomal degradation of proteins, mediated by multi-ubiquitin chains (Terrell et al., 1998). Upon ligand binding to Ste2p, the receptor is phosphorylated. This has been shown to be required for the ubiquitination of the receptor and consequently its internalisation and downregulation (Hicke et al., 1998). This process demonstrates that ligand-induced downregulation of its own receptor can act as a mechanism of limiting the activity of ligands.

Ubiquitination dependent internalisation and downregulation of cell surface proteins has also been observed in mammalian cells for a variety of ligand-receptor pairs (Shtiegman

and Yarden, 2003). As described previously, ubiquitination of the TGFβ type 1 receptor by Smurf ubiquitin ligases mediates the degradation of both the receptor and ligand (Ebisawa et al., 2001; Kavsak et al., 2000) (Zhu et al., 1999).

5.2.2 The role of ubiquitination of Arrow in Wingless degradation

A significant portion of Wingless degradation occurs in a lysosomal compartment, as mutant clones of *deep-orange* accumulate Wingless (L. Dubois personal communication). It is possible that ubiquitination of Arrow could target the ligand-receptor complex to degradation. Deletion of the lysine residue in the cytoplasmic tail of the β_2 – Adrenergic receptor, which is degraded following ubiquitination, prevents receptor degradation (Shenoy et al., 2001). I carried out similar mutations on Arrow and created a form of Arrow where the seven lysine residues present in the cytoplasmic tail are mutated to alanines (hereby referred to as ArrowΔLysine) and presumably cannot be ubiquitinated (Figure 5.4a). Arrow ΔLysine was tagged with the HA epitope and introduced into flies.

When this form of Arrow is overexpressed using *dpp-gal4*, no effect on Wingless distribution is observed (Figure 5.4b-b'). When ArrowΔLysine is expressed together with Dfz2 full-length using *dpp-gal4*, Wingless is still stabilised, suggesting that ArrowΔLysine does not degrade Wingless (Figure 5.4c-c''). This is consistent with the idea that ubiquitination could be a modification that regulates degradation of Arrow and consequently Wingless.

Arrow ΔLysine localises to the basolateral surface of the imaginal disc cells in a similar manner to Arrow full-length and colocalises with Dfz2 in this domain (Figure 5.4d-d''). Suggesting that localisation of Arrow is unaffected in the absence of ubiquitination.

Ubiquitination of Arrow could control its endocytosis and consequently its ability to target Wingless for degradation. I therefore investigated whether ArrowΔLysine

colocalised with Wingless in endosomes. ArrowΔLysine was expressed with *ap-gal4* and endosomes labelled with fluorescent Dextran. Of the punctate Wingless structures, only 1.6% of those observed colocalise with Dextran and ArrowΔLysine (Figure 5.5 and Table 5.1). Suggesting that ubiquitination could be required for Arrow endocytosis. Wingless does however still colocalise with dextran in endosomes (Arrows in Figure 5.5b-b'''). Indicating that ArrowΔLysine does not block Wingless endocytosis.

Interestingly, ArrowΔLysine levels appear to be elevated in the cells closest to the Wingless producing cells (Figure 5.5a). This is observed reproducibly when ArrowΔLysine is expressed using Ap-gal4, However, it is not observed when ArrowΔLysine is expressed using Dpp-gal4.

Figure 5.4 ArrowΔLysine does not degrade Wingless

(a) The lysine residues present in the cytoplasmic tail of Arrow (highlighted in red), to make ArrowΔLysine, each Lysine residue was mutated to an Alanine.
(b-b') Wing disc of the genotype *dpp-gal4 UAS-ArrowΔLysine* stained with anti-Wingless (red) and anti-HA (green). Wingless distribution is unaffected.
(c-c'') Wing disc of the genotype *dpp-gal4 UAS-dfz2-FLAG UAS-ArrowΔLysine* stained with anti-Wingless (red), anti-HA (green) and anti-FLAG (blue). Wingless is stabilised by Dfz2, even in the presence of ArrowΔLysine.
(d-d'') Wing disc of the same genotype as (c-c'') labelled with anti-HA (green (d and d')) and anti-FLAG (red (d and d'')) Arrow-ΔLysine is present in the basolateral domain where it colocalises with Dfz2, which is present in both the apical and basolateral domains.
Images are basolateral single confocal sections.

TRIGKSRTEPKDDQATDPLSPSTLSKSQRVSKIASVADAVRMS
TLNSRNSMNSYDRNHITGASSSTTNGSSMVAYPINPPPSPATR
SRRPYRHYKIINQPPPPTPCSTDICDESDSNYTSKSNSNNSNG
GATKHSSSSAAACLQYGYDSEPYPPPPTPRSHYHSDVRIVPES
SCPPSPSSRSSTYFSPLPPPPSPVQSPSRGFTZHFESYMZ

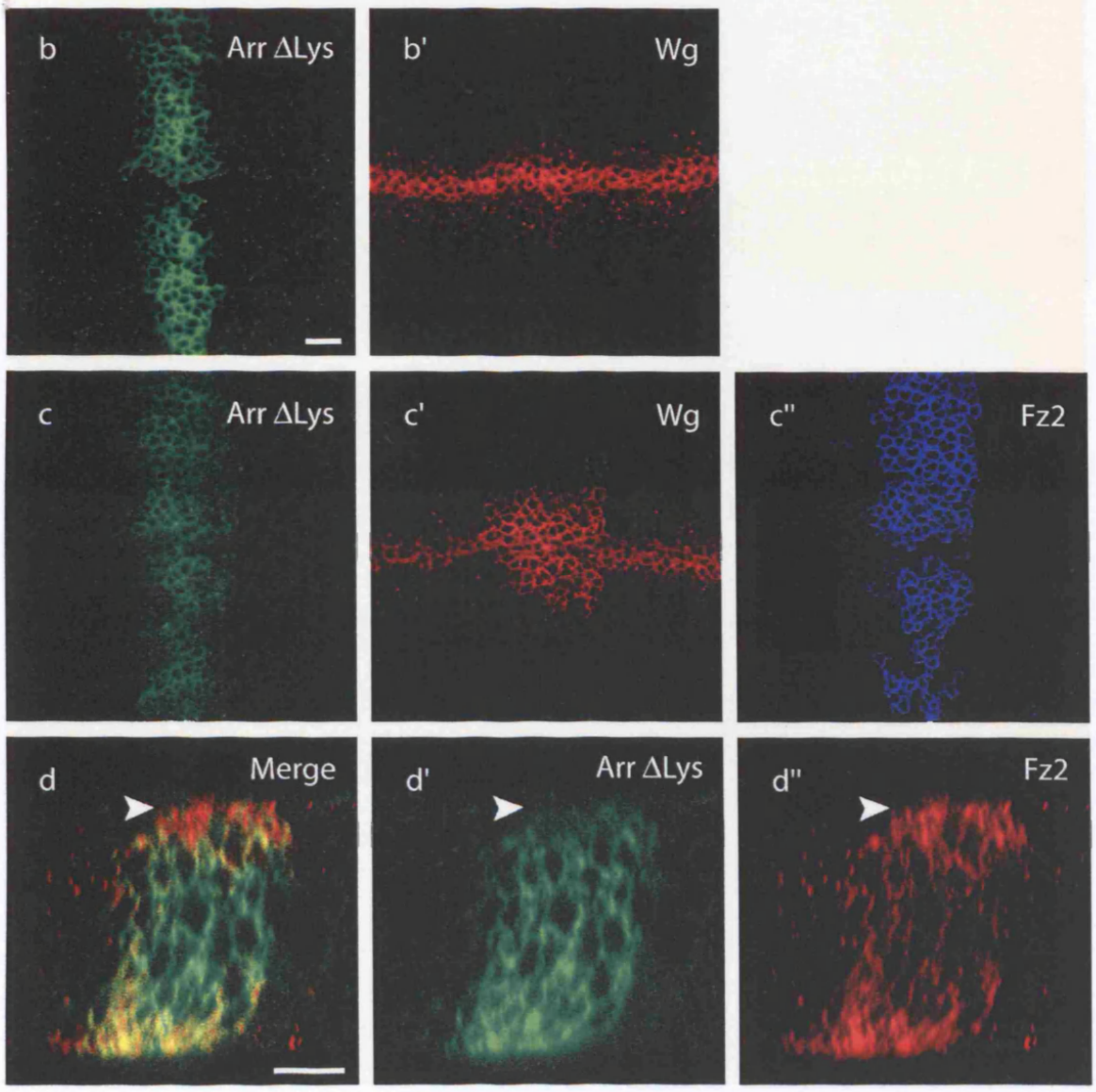
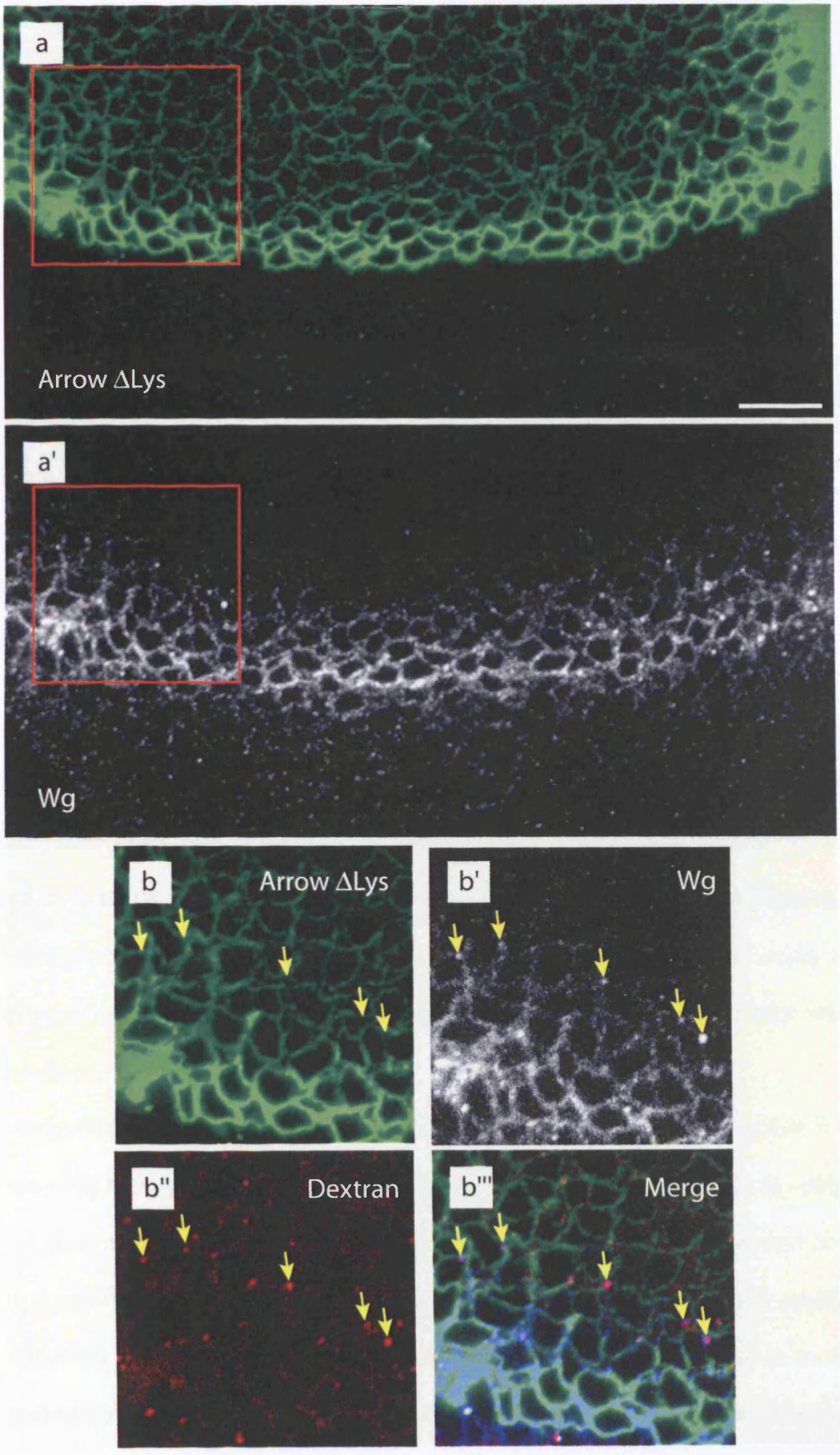


Figure 5.5 ArrowΔLysine is not internalised with Wingless

Wing disc of the phenotype *ap-gal4 UAS-Arrow-Δlysine,* labelled with dextran-Texas Red (red) and stained for anti-Wingless (white) and anti-HA (green).
(a) Punctate Wingless structures are observed in the domain of overexpression.
(b-b''') High magnification of the boxed area in (a). The punctate Wingless structures colocalise with dextran but not with Arrow-ΔLysine, indicating that Arrow-Δlysine is not internalised, but Wingless is.
Images are basolateral single confocal sections.


a
Arrow ΔLys
a'
Wg
b
Arrow ΔLys
b'
Wg
b''
Dextran
b'''
Merge

	Percentage Colocalisation of Arrow, Wingless and Dextran
Arrow full-length	52.48%
Arrow-PPAP	48.10%
Arrow ΔLysine	1.61%

Table 5.1 Percentage colocalisation between Wingless, Dextran and Arrow in Wingless punctate structures.

5.2.3 ArrowΔLysine does not ectopically activate signalling

ArrowΔLysine does not appear to be able to target Wingless bound to Dfz2 to degradation, it might therefore be expected that overexpression of ArrowΔLysine would lead to ectopic signalling. However, when ArrowΔLysine is expressed using the *ap-gal4* driver no ectopic signalling effects are observed and the flies appear wild-type (Figure 4.6a-a').

My investigations into ArrowΔLysine suggest it cannot perform any of Arrow's normal functions, even though it is localised to the basolateral surface of discs, as is Arrow full-length. It does not degrade Wingless efficiently, it is not endocytosed and it cannot activate signalling. This suggests that removal of the lysines severely undermines Arrow function and consequently it is difficult to draw firm conclusions as to whether the ubiquitination is important for endocytosis or degradation of Arrow or Wingless.

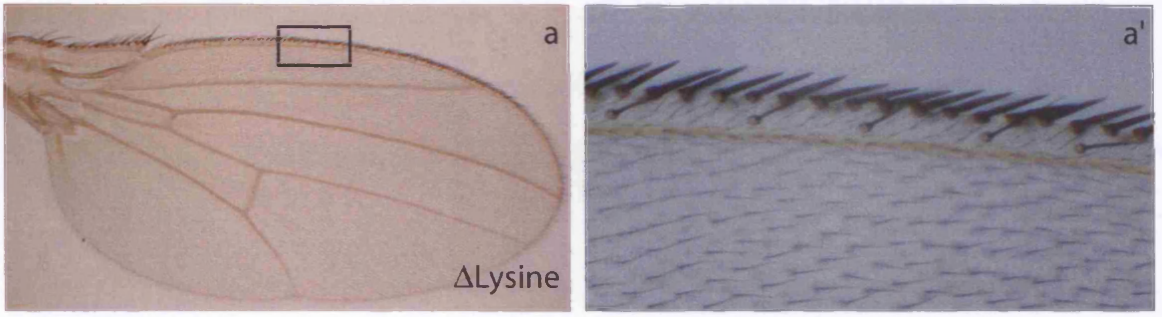


Figure 5.6 Ectopic expression of ArrowΔLysine does not affect adult wings

Wing of the genotype *ap-gal4 UAS-Arrow-ΔLysine* (a). High magnification of the boxed area in (a) shown in (a'). Wings are wild-type in appearance.

5.3 Summary

- Mutation of the PPP(S/T)P motifs prevents Arrow from degrading Wingless bound to Dfz2.
- Arrow-PPAP colocalises with Wingless in endocytic structures.
- Arrow-PPAP blocks Wingless signalling.
- Mutation of the lysines in the cytoplasmic tail of Arrow prevents normal Arrow function.

The results in this chapter suggest that phosphorylation is required in order for Arrow to trigger Wingless degradation. Preventing the phosphorylation of Arrow prevents degradation of Wingless bound to Dfz2. Phosphorylation of Arrow is not required for Wingless internalisation as Wingless is still internalised with Arrow-PPAP. When overexpressed, Arrow-PPAP reduces the size of the wing and also causes the loss of dorsal margin bristles, indicating a reduction in Wingless signalling and suggesting that Arrow-PPAP acts as a dominant negative on Wingless signalling.

As phosphorylation of Arrow is required for Wingless degradation, this adds another level of control to the mechanism of signalling and degradation. Preventing Wingless degradation in the absence of Arrow means that Wingless can only be degraded after a signalling complex has been formed and Arrow has been phosphorylated. The kinases that phosphorylate LRP6 have recently been identified and it will be interesting to assess Wingless degradation in the absence of these components (Davidson et al., 2005; Zeng et al., 2005).

It is not yet clear whether ubiquitination of Arrow is required for Wingless degradation. While deletion of the internal lysines in Arrow appears to block Wingless degradation, this form of Arrow is unable to localise to endosomes and importantly does not activate

signalling. Therefore, the reason that this form of Arrow cannot direct Wingless to degradation may be due to indirect effects that prevent it from associating with Wingless or Dfz2. Expression of ArrowΔLysine with Ap-gal4 indicates that ArrowΔLysine levels are elevated where Wingless signalling is at its highest. It is possible that ArrowΔLysine is stabilised in the presence of Wingless.

Further investigation is required in order to characterise the potential role of ubiquitination of Arrow in Wingless trafficking.

CHAPTER 6 – DISCUSSION

6 CHAPTER 6 - DISCUSSION

Ligand capture, transport and degradation all contribute to the regulation of the distribution of extracellular ligands and the receptors can play key roles in each of these processes. The mechanisms of Wingless transport and capture by the receptors have been extensively studied, however little is known about the degradation of Wingless. Previous work identified lysosomal degradation as important in the regulation of Wingless distribution in the embryo (Dubois et al., 2001), but it was not known how the receptors impact on this process. Moreover, the role of Wingless degradation in the wing disc had not been investigated. Receptor-mediated degradation occurs in three steps, capture, endocytosis and targeting to lysosomes. Previous work has suggested that Wingless is primarily captured by Dfz2, the aim of this study was to identify how the next two steps, endocytosis and targeting to lysosomes, are mediated by the receptors.

The main conclusions of my work are as follows:

- Dfz2 mediates Wingless endocytosis; signals in the cytoplasmic tail and the cytoplasmic loops are required for Wingless endocytosis.
- Arrow may also contribute to endocytosis and importantly; it also provides the degradation signal that targets Wingless bound to Dfz2 to lysosomes.
- Motifs in the cytoplasmic tail of Arrow appear to mediate the degradation of Wingless bound to Dfz2. Phosphorylation of the cytoplasmic tail of Arrow appears to be required for degradation.

Table 6.1 Phenotypes associated with Dfz2 constructs and Dally-like

	Stabilise Wg	Ectopic Signalling	Stabilisation Repressed by Arrow	Internalised with Wg
Dfz2	+	+	+	+
Dfz2AV	+	+	+	+
Dfz2-GPI	+	DN	-	-
Dally-like	+	DN (1)	- (2)	*

DN – Dominant negative
(1) (Kirkpatrick et al., 2004)
(2) A reduction in Wingless stabilisation by Dally-like is observed, however Wingless is still substantially stabilised, unlike in the case of Arrow and full-length Dfz2.
*Internalisation of Wingless with Dally-like has not been assessed

Table 6.2 Phenotypes associated with Arrow constructs

	Stabilise Wg	Ectopic Signalling	Represses Dfz2 stabilisation	Internalised with Wg
Arrow	-	+	+	+
Arrow-PPAP	weak	DN	-	+
Arrow ΔLysine	-	-	-	-
Arrow ΔC	weak	-	-	

DN – Dominant negative

Table 6.3 Phenotypes observed in mutant clones

	Stabilise Wg	Wg internalised
Arrow	+	+
fz Dfz2	-	+ (1)
Dsh	+	+

(1) (Baeg et al., 2004)

6.1 Wingless Endocytosis

In this work I have examined Wingless endocytosis and the results suggest that both Dfz2 and Arrow can contribute to this process. Dfz2 colocalises with Wingless in Dextran-positive structures and truncating Dfz2 reduces this colocalisation. The internalisation of Dextran has been previously used in a number of studies to observe endocytosis in the wing disc (Entchev et al., 2000). While the data are compelling, I am aware of the limitations of the dextran technique. The colocalisation observed between Dfz2, Wingless and dextran does not prove that they were internalised together, it is possible that both were internalised independently but later came together in an endosome. A possible improvement here would be to use antibodies that recognise specific endocytic compartments.

In parallel to this work, Eugenia Piddini has investigated Wingless endocytosis in cell culture. *Drosophila* S2 cells transfected with Dfz2 endocytose Wingless, whereas cells transfected with Dfz2-GPI, which I found reduces Wingless endocytosis in discs, do not.

I have provided evidence that Arrow may also contribute to endocytosis. This is based on the finding that Arrow is able to degrade Wingless bound to Dfz2AV, a form of Frizzled that is less efficient than full-length Dfz2 at Wingless internalisation. However, Dfz2AV can still internalise Wingless, albeit less efficiently, and this might be sufficient to allow Arrow to mediate Wingless degradation. In support of a possible role for Arrow in Wingless endocytosis, S2 cells transfected with Arrow stimulate the endocytosis of Wingless (E. Piddini, Personal Communication), suggesting that Arrow can indeed endocytose Wingless.

Dsh has been implicated in the internalisation of Frizzled proteins upon stimulation by Wnts (Chen et al., 2003), suggesting that Dsh could also mediate Wnt internalisation.

However, I find that Wingless can still be internalised in the absence of Dsh. This could be because Dsh is required for Fz internalisation but not essential for Wingless internalisation, implying that Frizzled-independent endocytosis of Wingless can occur. Indeed, a recent study found that Wingless internalisation occurs in the absence of Fz and Dfz2 (Baeg et al., 2004), suggesting that alternative mechanisms for Wingless internalisation must exist. It would be interesting to address whether Dfz2 endocytosis can occur in the absence of Dsh. This experiment is complicated by the lack of an antibody that recognises endogenous Dfz2. However, it could be addressed by creating clones of cells that lack Dsh and ectopically express tagged Dfz2 using the MARCM system. Alternatively, endocytosis of Dfz2 and Wingless in cells that lack Dsh could be investigated in cell culture and this could identify whether Dsh is necessary for Wingless and Dfz2 endocytosis in *Drosophila* cells.

Together, my results and the cell culture studies suggest that both Arrow and Dfz2 can contribute to Wingless endocytosis. However, Wingless internalisation can occur in the absence of Fz and Dfz2 (Baeg et al., 2004) and Arrow (my own findings). In the absence of Fz and Dfz2, it is possible that Arrow could compensate and vice versa. In order to investigate whether this is the case, Wingless internalisation in the absence of Fz, Dfz2 and Arrow could be analysed. Due to the genetic location of the three genes, this is not straightforward in discs, but could be addressed in cell culture.

6.2 Wingless degradation

Endocytosis by Dfz2 is clearly not sufficient for degradation, as ectopic expression of Dfz2 leads to stabilisation of Wingless. Still, reducing the ability of Dfz2 to internalise Wingless increases the amount of Wingless stabilised, suggesting that Dfz2 does participate in degradation. I reasoned that Dfz2-mediated endocytosis is not sufficient for degradation because another factor could be required for targeting to lysosomes after endocytosis. Ectopic expression of Arrow in tandem with Dfz2 prevents Wingless

stabilisation by Dfz2, suggesting that the limiting factor could be Arrow. Truncating the cytoplasmic tail of Arrow prevents it from removing Wingless bound to Dfz2. Furthermore, clones that lack Arrow accumulate Wingless, suggesting that indeed Arrow is required for Wingless degradation. Arrow is unable to efficiently degrade Wingless bound to either Dfz2-GPI or Dally-like, suggesting that Arrow can only interact with full-length Dfz2 to bring about Wingless degradation. Dally-like, Dfz2-GPI and Dfz2 full-length stabilise Wingless in the basolateral domain, where Arrow is also localised, suggesting that the reason that Arrow cannot efficiently degrade Wingless bound to Dally-like and Dfz2-GPI is not due to different localisation of the respective receptors.

It is possible that the inability of Arrow to remove Wingless bound to Dally-like may be due to the strength of the interaction between Wingless and Dally-like. The binding affinity of Wingless to Dfz2 has been determined but as yet, the Wingless-Dally-like binding affinity has not been assessed. However, it is possible that if the binding between Wingless and Dally-like is substantially stronger than that of Wingless-Dfz2, and therefore Arrow is unable to remove Wingless bound to Dally-like.

Work in *Xenopus* has identified Kremen proteins as negative regulators of Wnt signalling. Kremens act by stimulating LRP endocytosis and possibly degradation (Mao et al., 2002). It remains to be seen whether this leads to degradation of Wnt during *Xenopus* embryogenesis. Moreover, no Kremen homolog has been identified in *Drosophila*, suggesting that alternative mechanisms must exist to stimulate endocytosis and degradation of Wingless and Arrow in the fly. Further work is clearly needed to understand the mechanism that leads to Wingless degradation by Arrow in the wing disc.

The requirement for Dsh in degradation of Wingless is unclear; it is not essential for Wingless endocytosis, although cells that lack Dsh do accumulate Wingless, suggesting

a possible role in degradation. The role of Dsh in degradation of Wingless by Arrow and Dfz2 could be addressed by using the MARCM technique to create clones of cells that ectopically express Arrow and Dfz2 but lack Dsh. Clones that express Arrow and Dfz2 would be expected to have near wild type levels of Wingless, If Dsh were required for degradation, then removing it could cause Wingless accumulation in these cells.

The observation that *arrow* mutant clones accumulate Wingless is evidence that Arrow is required for degradation of Wingless. However, *deep-orange* mutant cells, accumulate Wingless to an even higher level than *arrow* mutants (Personal communication with L. Dubois). This suggests Arrow-mediated degradation is not the only mechanism that leads to lysosomal degradation of Wingless. My finding that Arrow cannot efficiently degrade Wingless bound to Dally-like suggests that an alternative mechanism may operate to remove Wingless bound to proteoglycans.

6.3 Model of Wingless degradation

My findings suggest a model whereby the two receptors contribute distinct, though overlapping trafficking activities that, together lead to the degradation of Wingless (Figure 6.1). Dfz2 is the primary receptor for Wingless capture. Upon capture by Dfz2, Wingless is endocytosed. This is consistent with the observation that Fz4 is endocytosed upon stimulation by Wnt5a (Chen et al., 2003). Endocytosis is clearly insufficient for degradation, as expression of Dfz2 leads to Wingless stabilisation. In this work, I suggest that Arrow provides the signal that targets Wingless captured by Dfz2 to a lysosomal compartment. Signals in the cytoplasmic tail of Arrow appear to be required to target Wingless for degradation. As I have shown, phosphorylation of the PPP(S/T)P motifs is also required for degradation.

Previous work has suggested that Dfz2 acts to protect Wingless from degradation (Cadigan et al., 1998); this idea was mainly based on the observation that overexpression of Dfz2 causes the accumulation of Wingless. The work presented here

suggests that in the presence of Arrow, Dfz2 plays a key role in Wingless degradation. I find that Dfz2 mediates Wingless endocytosis and furthermore, as reducing Dfz2 endocytosis increases its ability to stabilise Wingless, endocytosis by Dfz2 appears to be required for Wingless degradation. Under conditions of overexpression of Dfz2, Arrow becomes limiting and, in the absence of a degradation signal, Wingless accumulates. Dfz2 therefore contributes to degradation by capturing and endocytosing Wingless, but is not sufficient for it. As Arrow has a low affinity for Wingless (Wu and Nusse, 2002) compared to Dfz2, it is likely to require Dfz2 to facilitate binding (Cong et al., 2004), ensuring that a signalling complex of Wingless, Dfz2 and Arrow are assembled prior to degradation.

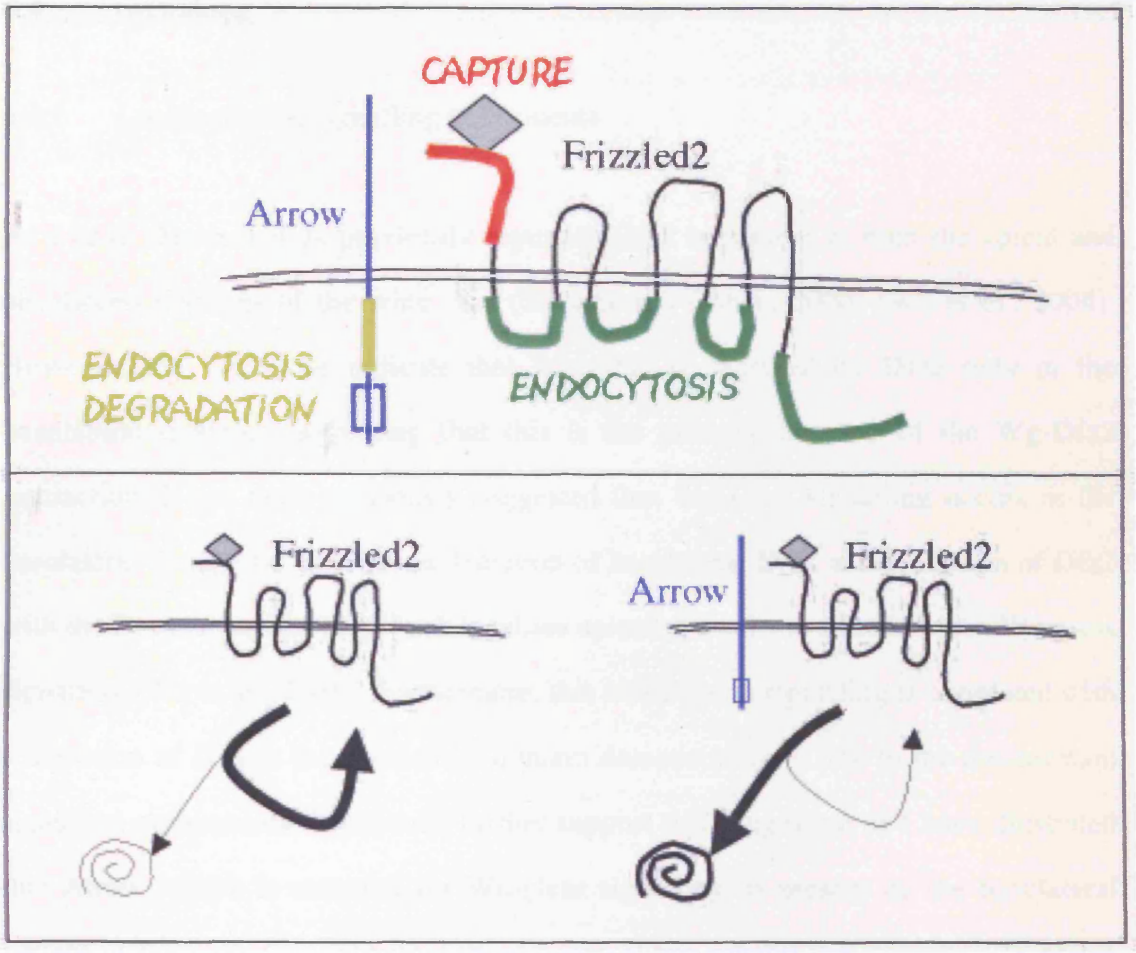


Figure 6.1 Model of Wingless degradation

Top: Dfz2 provides most of the capturing activity while Arrow brings a degradation signal. Both receptors provide an internalization signal. Dfz2 internalises Wingless in the absence of Arrow. Arrow can also possibly contribute to endocytosis.

Bottom: When Dfz2 is in relative excess at the cell surface, Wingless is captured and put on hold (possibly protected from putative extracellular proteases) until Arrow becomes available to signal and trigger degradation.

6.4 Signalling

6.4.1 Localisation of signalling components

As I have shown and as previously reported, Dfz2 is present in both the apical and basolateral domains of the wing disc (Strigini and Cohen, 2000) (Wu et al., 2004). However, the z-sections indicate that Wingless is captured by Dfz2 only in the basolateral domain, suggesting that this is the primary location of the Wg-Dfz2 interaction. It has been previously suggested that Wg-Dfz2 signalling occurs in the basolateral domain of the disc, as depletion of basolateral Dfz2 using a fusion of Dfz2 with the Fz cytoplasmic tail (which localises apically) results in a reduction in Wingless signalling (Wu et al., 2004). Furthermore, this reduction in signalling is correlated with a depletion of Dsh in the basolateral domain demonstrating a link to the downstream signalling components. My results further support this suggestion as I have illustrated that Arrow, which is essential for Wingless signalling, is present on the basolateral surface of the wing disc. Together these observations suggest that canonical Wingless signalling does indeed occur in the basolateral domain, as Wg, Dfz2, Arrow and Dsh are all localised here, as is Dally-like(Kreuger et al., 2004).

6.4.2 The requirement for signalling

In the absence of either Arrow or Dsh, Wingless can be endocytosed, illustrating that signalling is not required for Wingless endocytosis. However, preventing Arrow phosphorylation prevents it from degrading Wingless, suggesting that signalling is required for degradation. Signalling is, however, not sufficient for degradation following endocytosis by Dfz2, as ectopic expression of activated Armadillo does not remove Wingless bound to Dfz2. Together, these observations suggest that upon binding to Dfz2, Wingless is internalised, and that subsequent association with phosphorylated Arrow, leads to degradation. This mechanism could ensure that all

Wingless captured by Dfz2 is only degraded after signalling has been initiated. The phosphorylation of Arrow could act as a docking site for a protein that would direct it, along with Dfz2 and Wingless, to degradation.

Is endocytosis required for Wingless signalling? A recent study has examined this question. Seto et al., (2006) used RNAi and dominant negative forms of trafficking components to reduce endocytosis or block endocytosis in *Drosophila* cells and imaginal discs. They found that Rab5 RNAi reduces a Wingless signalling readout in S2 cells (Seto and Bellen, 2006). However, the result is variable, dependent on the reporter construct used and the timing of the RNAi (DasGupta et al., 2005). Furthermore, in Wing imaginal discs, ectopic expression of *shibire* dominant-negative or Rab5 dominant-negative results in a reduction in the levels of Wg target genes (Seto and Bellen, 2006). However, these results must be treated with caution as in each of these experiments, the cells are likely to be impaired in a number of functions and the reduction in target gene levels may be a result of general reduction of gene expression in the cells. Indeed Engrailed, which is not known to be regulated by Wingless or another signal, is greatly reduced in the presence of *shibire* dominant-negative (Piddini et al., 2005).

The requirement for signalling prior to degradation is also observed for EGF and EGFR. Upon ligand binding to EGFR, the receptor is activated by phosphorylation of the cytoplasmic tail. This recruits c-Cbl, which promotes ubiquitination of the receptor, resulting in degradation of the receptor and ligand (Shtiegman and Yarden, 2003). As ubiquitination is dependent on the activation of the receptor, degradation cannot occur until signalling has been initiated. Similarly, my work suggests that degradation of Wingless is dependent on the recruitment of Arrow to the Wingless-Dfz2 complex and the phosphorylation of Arrow. Identification of Arrow binding partners may lead to the identification of the proteins that then target Wingless to degradation.

Interestingly, endocytosis plays both positive and negative roles in the modulation of Notch signalling. Notch signalling is notably different to that of Wg as it occurs in via

juxtacrine mechanism as both the ligand (Delta and Serrate) and the receptor (Notch) are membrane bound. Two cleavage events govern Notch signalling. Upon binding of Delta to Notch, the Notch extracellular domain is cleaved and is internalised with Delta into the signal sending cell, following this, the Notch cytoplasmic tail is cleaved and is subsequently directed to the nucleus in the signal receiving cell where it activates transcription (Struhl and Adachi, 1998) (Schroeter et al., 1998). Endocytosis of the Notch extracellular domain by Delta is essential for the activation of signalling, Delta mutant deficient for endocytosis do not activate signalling (Parks et al., 2000). The mechanism of Notch activation following Delta internalisation is not yet clear, however, it is possible that Delta endocytosis triggers the second cleavage event, leading to the activation of signalling(Parks et al., 2000). Endocytosis and degradation of Notch negatively regulates signalling. In *C.elegans* mutation of a dileucine motif in the cytoplasmic tail of Notch prevents Notch internalisation and degradation, leading to an increase in signalling (Shaye and Greenwald, 2002). In addition to the dileucine motif, studies in *Drosophila* suggest that ubiquitination may also regulate Notch downregulation. Suppressor of Deltex [Su(dx)] encodes an E3 ubiquitin ligase and is essential for Notch downregulation. However, a direct action of [Su (dx)] on Notch has yet to be demonstrated (Cornell et al., 1999; Fostier et al., 1998).

6.5 Why a two-receptor system for degradation?

The sharing of trafficking activities by two receptors could allow for the modulation of Wingless distribution in the wing disc. Wingless captured by Dfz2 is stabilised at the cell surface and in endosomes. As Dfz2 binds and stabilises Wingless, this could help to create a stable distribution of Wingless in the disc. As Arrow brings both the signalling motif and the degradation motif to the complex, it could provide an on-off switch that can regulate signalling levels in the receiving cells. Coupling signalling to degradation could ensure that signalling levels are limited and the modulation of Arrow levels in the cells would therefore regulate the signalling and degradation rates in each cell.

A two-receptor system of ligand trafficking is also utilised by TGFβ. In this system type II receptor binds the ligand and this is followed by the formation of a tripartite complex with type I receptor, which leads to the activation of signalling (Massague, 1998). Type I receptors, like Arrow, bring the degradation signal. Ubiquitination of type I receptors by Smurf proteins results in the degradation of both the ligand and the receptor (Ebisawa et al., 2001; Kavsak et al., 2000). This mechanism allows the receptors to direct the ligand towards degradation and signalling appropriately (Di Guglielmo et al., 2003). It may be no coincidence that both Dpp (the fly TGFβ) and Wingless, which can both act over a relatively long distance, use two receptors for signalling and degradation. It is possible that separation of capture and degradation is a feature required for long-range signalling.

Two transmembrane proteins also function in the transduction and trafficking of the Hh signal, albeit in a very different way to Wg and Dpp. In the absence of Hh, the seven pass transmembrane receptor Smo is kept in an inactive state by Ptc (Murone et al., 1999). Upon binding of Hh to Ptc, the inhibition is released and Smo interacts with the downstream components to activate the signalling pathway. In addition to its roles in Smo inhibition and Hh binding, Ptc also regulates the trafficking of the Hh ligand. The

dynamin-dependent endocytosis of Hh by Ptc has been demonstrated in both vertebrates and *Drosophila* wing discs presumably leading to its targeting to lysosomes (Incardona et al., 2000) (Torroja et al., 2004). In addition to its role in downregulation of the signal, endocytic trafficking also appears to play a role in the process of Smo activation.

Upon addition of Hh, Smo and Ptc are readily endocytosed into early endosomes here they segregate, suggesting a mechanism whereby the differential sorting of the two transmembrane receptors results in the relief of the inhibition of Smo (Incardona et al., 2002). However, in *Drosophila* wing discs, endocytosis of Ptc does not appear to be essential for signalling. A Ptc mutant that is defective for endocytosis is still able to activate Hh signalling (Torroja et al., 2004). The results of Seto et al., (2006) have suggested that in contrast to the Hh pathway, endocytosis is essential for Wingless signalling. However, as stated previously, these results made the use of dominant negative components of the endocytic pathway, which may have had indirect effects on the readouts of signalling, used (Piddini et al., 2005). More specific assessment of the effect on Wingless signalling alone is required. I find that Dfz2AV, which is less effective at the endocytosis of Wingless, is still able to activate signalling. However, Wingless endocytosis is not completely blocked in the presence of Dfz2AV. The identification of the specific motifs required for the endocytosis of Dfz2 will allow for the separation of signalling and endocytosis to be more accurately assessed.

6.6 Future Work

During the completion of this work, a study was published examining the role of the Wingless receptors in the formation of the extracellular gradient of Wingless (Han et al., 2005). In this study the authors discovered, as I have, that in *arrow* mutant clones extracellular Wingless is accumulated. However, in conflict with my results, they also observe accumulation of extracellular Wingless in *fz, Dfz2 clones.* Furthermore, the

observation by Han et al., has been confirmed by Eugenia Piddini who has observed that Wingless accumulates in *fz [P21], Dfz2 [C1]* clones.

The reason for this difference could be due to the different alleles of *fz* used. I used the *fz [H51]* allele that is truncated before the final transmembrane domain and is a null for signalling, but in the Han Paper, they used the *fz [P21]* allele, which is truncated before the first transmembrane domain (Jones et al., 1996). Residual activity of the *fz [H51]* allele could account for the lack of Wingless accumulation I observe in *fz Dfz2* clones.

Importantly, the authors suggest that the Wingless accumulation observed in both *arrow* and *fz, Dfz2* clones is not due to reduced degradation, but is due to an upregulation of Dally-like, which stabilises Wingless (Han et al., 2005). My gain of function data clearly supports a function for Arrow in targeting Wingless to degradation. However, it is possible that stabilisation by Dally-like also contributes to Wingless accumulation in *arrow* mutant clones. In order to investigate this possibility, one could assess Wingless distribution in *arrow* mutant clones that also ectopically express activated Armadillo. As activated Armadillo stimulates Wingless signalling, Dally-like will be repressed. If Wingless levels are still elevated in the clones, it will indicate that the accumulation observed in *arrow* clones is not only due to increased levels of Dally-like.

The experiments I carried out to investigate the possible role of ubiquitination of Arrow in mediating Wingless degradation were inconclusive. The deletion of the lysine residues appeared to prevent Arrow from carrying out all of its normal functions. However interestingly, when expressed with ap-Gal4 ArrowΔLysine levels are elevated close to the domain of Wingless expression, a phenotype not observed with the other forms of Arrow. Suggesting a possibility that in the presence of Wingless ArrowΔLysine is stabilised

Therefore, a new approach is required to address whether Arrow ubiquitination mediates Wingless degradation.

My work has suggested a mechanism by which Wingless is degraded in the wing imaginal disc of *Drosophila*. It remains to be seen how Wingless degradation is

regulated by the receptors in the embryo. As endocytosis and degradation underpin the asymmetric distribution of Wingless after stage 11, it is possible that Arrow and Dfz2 could contribute to this process. Indeed, *Dfz2* mutants exhibit elevated levels of Wingless protein (Moline et al., 2000). However, in *arrow* mutant embryos Wingless distribution appears normal (Wehrli et al., 2000). Therefore, the contribution of Arrow and Dfz2 to Wingless degradation in the embryo requires further investigation.

7 APPENDIX

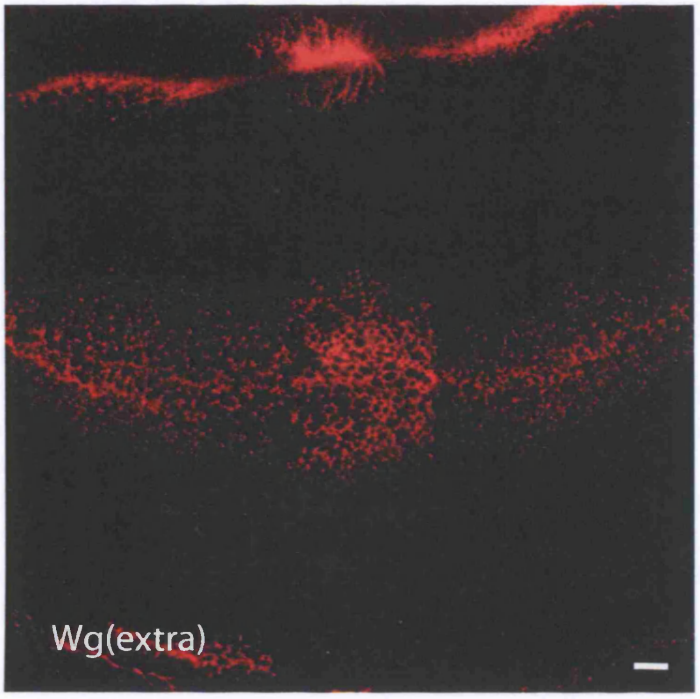


Figure A1 Wg is stabilised extracellularily by Dfz2

Wing disc of the genotype DppGal4 UASDfz2-FLAG. Labelled with anti-Wingless using an extracellular staining protocol. Wingless accumulates extracellularily (see also Strigini and Cohen 2000).

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